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Review

Physiological effects of sustained blockade of excitatory synaptic transmission on spontaneously active developing neuronal networks—an inquiry into the reciprocal linkage between intrinsic biorhythms and neuroplasticity in early ontogeny

M.A. Corner[a,b,*], J. van Pelt[a,b], P.S. Wolters[a,b], R.E. Baker[a,b], R.H. Nuytinck[a,b]

[a]*Academic Medical Centre, Meibergdreef 33, Netherlands Institute for Brain Research, 1105 AZ Amsterdam, The Netherlands*
[b]*Neuroscience Research School, University of Amsterdam, Amsterdam, The Netherlands*

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Abstract

Spontaneous bioelectric activity (SBA) taking the form of extracellularly recorded spike trains (SBA) has been quantitatively analyzed in organotypic neonatal rat visual cortex explants at different ages in vitro, and the effects investigated of both short- and long-term pharmacological suppression of glutamatergic synaptic transmission. In the presence of APV, a selective NMDA receptor blocker, 1–2- (but not 3-) week-old cultures recovered their previous SBA levels in a matter of hours, although in imitation of the acute effect of the GABAergic inhibitor picrotoxin (PTX), bursts of action potentials were abnormally short and intense. Cultures treated either overnight or chronically for 1–3 weeks with APV, the AMPA/kainate receptor blocker DNQX, or a combination of the two were found to display very different abnormalities in their firing patterns. NMDA receptor blockade for 3 weeks produced the most severe deviations from control SBA, consisting of greatly prolonged and intensified burst firing with a strong tendency to be broken up into trains of shorter spike clusters. This pattern was most closely approximated by acute GABAergic disinhibition in cultures of the same age, but this latter treatment also differed in several respects from the chronic-APV effect. In 2-week-old explants, in contrast, it was the APV + DNQX treated group which showed the most exaggerated spike bursts. Functional maturation of neocortical networks, therefore, may specifically require NMDA receptor activation (not merely a high level of neuronal firing) which initially is driven by endogenous rather than afferent evoked bioelectric activity. Putative cellular mechanisms are discussed in the context of a thorough review of the extensive but scattered literature relating activity-dependent brain development to spontaneous neuronal firing patterns. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Explant cultures; Visual cortex; Spontaneous bioelectric activity; Spike-train analysis; Burst firing; NMDA receptors; AMPA receptors; GABA-ergic inhibition; Minute-order rhythms; Neural network formation

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* Corresponding author. Address: Academic Medical Centre, Meibergdreef 33, Netherlands Institute for Brain Research, 1105 AZ Amsterdam, The Netherlands. Fax: +31-20-696-1006.
E-mail address: m.corner@nih.knaw.nl (M.A. Corner).

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1. Background considerations

In the early 1960s, two basic physiological processes were discovered which, in almost half a century which has elapsed since then, have not hitherto been comprehensively reviewed in conjunction with one another (but see Refs. [52,134,208,485,509,654] for preliminary attempts in that direction). The first discovery, which we will call *neuroplasticity*, involved the phenomenon that visually responsive neurons in the kitten striate cortex, although unexpectedly showing a high degree of selectivity even prior to visual experience, required non-specific stimulation of retinal origin for the maintenance and further development of this pre-existing functional organization ([319,749]; for later coverage see Refs. [44,519,620,630,643–645, 683,776]). At about the same time, it was discovered that the early embryonic central nervous system (CNS) is not only capable of reacting to sensory stimuli but also is an intrinsic *biorhythm* generator of neuromotor activity; indeed, excitatory synaptic interactions within the developing brain and spinal cord are at first almost completely of endogenous rather than reflexive origin ([128–131,141, 287–290,618]; for later coverage, see Refs. [20,49,133, 154,169–172,298,299,342,343,508–513,522,572,670,671, 715]). Not long afterwards, the persistence into later life of a special form of such brain 'self-stimulation' was demonstrated in the form of what came to be called 'active, c.q. REM, sleep' [337,580,710] and its neurophysiological basis soon began (and continues to this day) to be intensively analyzed [e.g. 14,338,456,598,730]. Comparative studies soon established that fundamentally similar 'primordial' neuromotor rhythms are a widespread, if indeed, not a universal feature of early vertebrate and invertebrate ontogeny [130,132,522]. Not long ago a survey of putative causal links between biorhythms and neural ontogeny would have been called for primarily as an attempt to stimulate interest in this theoretically extremely fundamental scientific question [134], but so much pertinent information has become available in the last decade or so that the need now is to bring some order into the welter of 'model' systems, experimental treatments and chemo-morpho-physiological assays scattered throughout the literature.

Spontaneous bioelectric activity (SBA) within the CNS, i.e. electrically detectable events originating within a neural network itself rather than being triggered by afferent input, is encountered throughout the brain as well as in the spinal cord (for complete inventarization see Section 5). It can take many forms, static as well as dynamic, ranging from the active maintenance of cell membrane properties [328,467, 468,527,566] to complex spatio-temporal patterns of synaptic and action potentials [1,19,87,140,147,159,162,271,291, 312,330,331,379,416,429,456,475,481,482,564,577,598, 666–675,678,697,702,723]. The latter are especially prominent at early stages of CNS development, and underlie primordial patterns of motility and brain function which, during the physiological state known as 'sleep', recur episodically throughout the life cycle (for ontogenetic review see Refs. [131,133]). On the basis of in vivo experiments, the neurophysiological activity associated with such movements has long been implicated in regulating the development of *spinal cord* motor systems (summarized in Refs. [134,522]), including the fine-tuning of selective connections between motoneurons and target muscles [101,102, 433,535]. A similar activity dependence was suspected for

the *cerebellum* as well [101,102] and, more recently, similar conclusions have been reached for developing *retinal* networks with respect to the role of SBA in the structural and functional innervation of forebrain visual areas [17,127, 295,534,558,590,620–622,643–645,696,742–744]. Even well-known neuroplastic effects of sensory stimulation (see above) have been shown to depend on the 'gating' or 'priming' effect of CNS sources of excitation [477,478, 642,646,647] which are at least partially of intrinsic origin.

With appropriate care, SBA can be well preserved in fragments of mammalian spinal cord or lower brainstem cultured in vitro and, if allowed to innervate muscle fibers, strikingly mimics the spontaneous motility patterns observed in utero [131,162]. Many 'higher' parts of the CNS have also proven to be spontaneously active—also in vivo (see above)—and typically generate very similar 'primitive' patterns of neuronal activity [133]; see Section 5. Tissue culture model systems have understandably been extensively employed, therefore, for analyzing normal physiological ontogeny and many of the factors involved in its regulation. The extensive but scattered literature in this field will be systematically reviewed in the present paper, using the most recent experimental results of our own research program spanning the last two decades as a 'scaffold' for inventarizing and interpreting the diverse mechanisms which are likely to be operative in activity-dependent maturational processes within the nervous system. The rationale all along (as in other labs, it goes without saying) has been, not to evade the necessity of eventually doing similar experiments under more natural physiological conditions, c.q. in the intact organism, but to facilitate the inevitable in vivo approach when the time is ripe by first surveying a wide range of putative causal factors, and establishing the most sensitive parameters for revealing their ontogenetic contribution. The relatively difficult and time-consuming whole animal experiments can then be limited to a focussed attack based on well formulated working hypotheses derived from the prior extensive 'screening' of suitable model systems. In this respect, too, the cell and tissue culture approach has proven to be valuable, inasmuch as computer simulation studies can sometimes approximate in vitro neural networks faithfully enough to put hypothetical mechanistic explanations of empirical phenomena to test [716–722] in a way which is simply not feasible for the brain as a whole. Finally, the study of isolated neuronal systems under controlled conditions can yield a wealth of information about developmental potentialities which either fail to be realized under normal conditions or which do occur and for which remedies are sought.

The earliest attempts to investigate the possible developmental significance of SBA, employing such tissue culture model systems to carry out pharmacological deprivation experiments, failed to reveal any activity-dependent effects on either the morphology or the physiology of the developing neurons (see Refs. [162,165]). An amazing degree of CNS maturation is evidently possible even in the virtually complete absence of bioelectric excitation, a conclusion subsequently confirmed by many in vivo studies [90,110, 111,292,293,298,299,304,464,560,604,605,622,729] as well as by increasingly refined in vitro analyses [27,30, 35,57,149,161,174,182–185,194,259,300,441,450,495, 551–555,588,603,613,614,653–659,712,713]. Many later experiments have shown, however, that a variety of more or less subtle disturbances of development can be expected in spinal cord networks following prolonged suppression of SBA if appropriate, and sufficiently sensitive, physiological or morphological assays are used [9,10,26–29,135,136,144, 212–214,273,313,314,463,653–659]. Since then, quite a few other CNS regions have been demonstrated to depend upon SBA for their full structural, functional and chemical maturation, at least under experimental conditions (see Section 5 for a thorough up-to-date survey of the literature).

Spontaneous activity patterns themselves have proven to be highly sensitive to perturbations in either the level or the pattern of action potential firing during neocortical network formation [134,149,503,551,553,554], presumably as a result of deviations from normal interneuronal connectivity patterns or intrinsic response properties—possibilities which will be discussed in the appropriate sections of this paper. This conclusion is based upon experiments in which dissociated/reaggregated cell cultures were chronically exposed either to the sodium channel blocker tetrodotoxin (TTX; [149,551]), thereby totally silencing the neurons as far as their spiking activity was concerned, or to the GABAa receptor blocker picrotoxin (PTX; [149,553]). After TTX was washed out, development appeared to have been frozen at a highly immature stage characterized by repetitive, more or less stereotyped, bursts of spikes. Chronic PTX treatment, in contrast, led to even less stereotyped bursting (when assayed in normal medium) than in control cultures of the same age, despite the fact that the two groups differed only in the patterning and not in the overall level of SBA. In both experiments, acute disinhibition transformed SBA from quasi-random firing to 'paroxysmal' spike bursts reminiscent of the primitive firing pattern [149,551], thereby suggesting a failure in the maturation of inhibitory synaptic control as the underlying factor in the 'hyper-activity syndrome' resulting from chronic suppression of SBA. Direct pharmacological and immuno-cytochemical evidence for weakened GABAergic activity in the TTX-treated cultures has strengthened this supposition [554,555], which is further supported by computer simulations of the effects of adding inhibitory units to a spontaneously bursting excitatory neural network [379]. Conversely, in relatively mature cord–muscle co-cultures from fetal rats, blocking synaptic inhibition converts irregular bursts at short intervals into stereotyped longer bursts in a pattern reminiscent of earlier developmental stages [674].

If SBA truly is a major causal factor in the functional maturation of the neocortex, it should be possible to affect that maturation in predictable ways by interfering with

naturally occurring excitatory neurotransmission. Our general research question can thus be reformulated as, what is the role of intrinsically generated excitatory synaptic drive in the ontogeny of neuronal network behavior, as expressed by the patterning of spontaneous action potential discharges? In particular, does its reduction lead to the formation of hyper-active networks and, if so, does such hyper-activity reflect *arrested* rather than *deviant* development? One means of eliminating synaptic transmission is by globally suppressing vesicle release and, since this is a calcium-dependent process, graded elevations of the extracellular magnesium ion concentration can achieve the desired degree of suppression [32,134,162,194,234,612]. A series of experiments preliminary to those reported in the present paper was carried out using this approach [137], and the results are in excellent agreement with the earlier TTX-induced effects (see above). This is all the more reassuring in view of the fact that 'organotypic' explants [25,31–38,583,587,588] were used in the former study instead of primary cultures. It was felt, namely, that this type of in vitro system would probably represent a step closer towards permitting a plausible extrapolation of the experimental findings to intact nervous systems was to be made. Although this assumption has turned out not to be justified in all respects (see later discussion), the mentioned confirmation with respect to the appearance of paroxysmal SBA patterns following prolonged functional deprivation is a welcome indication that this effect is not restricted to a single model system [724].

A more selective approach to depriving developing neural networks of excitatory interactions, and the one employed in most of the experiments to be reported here, is to chronically block glutamatergic synaptic transmission (this being the natural source of most of the excitatory drive in the neocortex [267,297,453]). Pharmacological means are available for selectively and reversibly blocking both the NMDA and the non-NMDA sub-classes of glutamate receptors (see Discussion sections) so we opted for the experimental strategy of eliminating both types of excitatory drive, separately as well as in combination. The latter approach proved to be an effective means of achieving a continuous near-total suppression of extracellularly measurable SBA over several weeks in vitro [134,503,724]. Selective NMDA receptor blockade, on the other hand, is of especially great interest in view of the crucial role which activation of precisely these receptors appears to play in a variety of developmental 'neuroplasticity' phenomena in vivo [44,45,179,263,295,296,462,547,548,562,621,627, 631–633,751,764]. There has been a serious difficulty in interpreting such effects, however, since no good control has been available until now for the drastic, non-specific reduction in network excitability which results at early stages from blocking APV-sensitive synaptic currents [8,42,54,79–81,134,255,256,316,326,333,341,356,367,406, 419,423,424,490,552,571,631,633,703,746,769]. Unless this factor is corrected for, no valid inferences can be made concerning a specific requirement for NMDA receptor activation per se during neural network formation [134,226, 295,344,345]. An empirical step towards solving this problem has been taken in the present investigation.

Our previous experiments examined a wide range of measures for quantifying spike trains in developing nerve culture systems [149,281,551–554]. Parameters that reflect the tendency for action potentials to be repetitively clustered in time have proven to be the most sensitive, both for developmental changes and for treatment effects. The basic neurophysiological activity pattern, characteristic for isolated cortex in situ as well as in vitro (see Section 5), is one of continually recurring bursts of spikes accompanied by field potentials representing summated synaptic activity [200], with each such burst lasting up to several hundred milliseconds and present in tight synchrony over all or most of the excitable portion of the explant (*network bursts*). Such discharges usually repeat themselves several times per minute with varying degrees of regularity, sometimes in trains of bursts ('activity periods': [128–133,145,287–290,522]) separated by one or more minutes of quiescence, but otherwise distributed evenly over time except for a certain 'jitter' in the interburst intervals and durations. The overall firing pattern, then, can be characterized by (i) the incidence of slow-wave events and the associated spike-bursts, (ii) the duration and mean intensity of spiking during each event, and (iii) the average firing rate and its fluctuations over the entire recording session.

Since network-burst events are commonly broken up into trains of shorter spike clusters (*mini-bursts*), at intervals which can vary greatly from one recording site to another (see Section 5), more than one criterion is often necessary for deciding what can most meaningfully be defined as a *cluster* of action potentials within a given spike train. Choosing objective criterion values for optimally making such multiple decisions is by no means a trivial question [118,347] and was addressed in the most recent experiments, reported in detail here, by locating the position of dips in the (often polymodal) interspike interval histograms computed from a large number of control spike trains. Certain of the interspike interval values found to reliably discriminate recurrent clusters from background 'noise' were highly consistent from one culture to the next, both for network-bursts and their component 'mini-bursts', so that these became the obvious criteria of choice for making intergroup comparisons of spontaneous spike clustering tendencies (see Section 2).

The above method for quantitatively characterizing the temporal organization of ongoing trains of action potentials thus supercedes our previous approaches, not because these have since proven to be unreliable—indeed, they have consistently led to the same conclusions whenever experiments were replicated (see below)—but as a result of our continual attempts to improve the power of spike-train analysis to reveal normal and deviant developmental

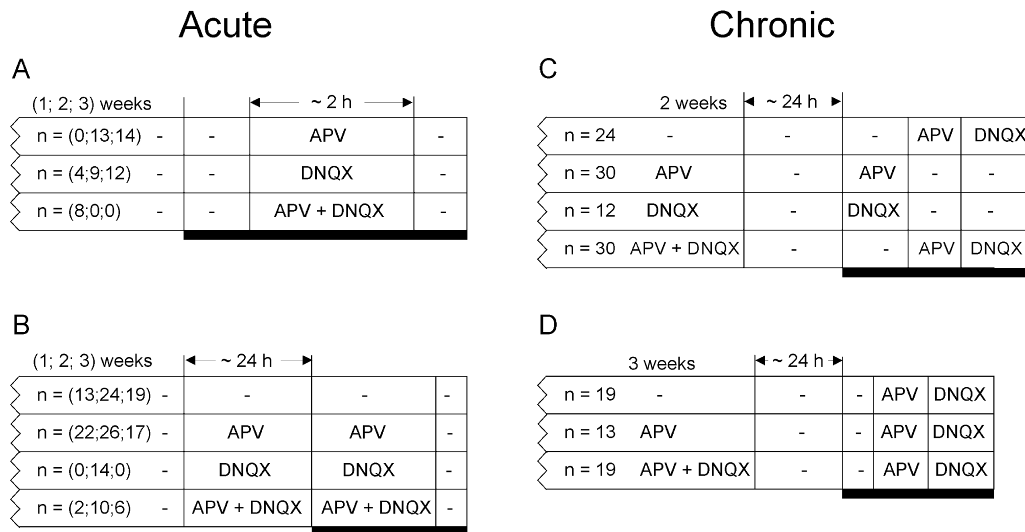

Fig. 1. Schematic overview of the experimental design for the acute and chronic treatment effects described in this paper see Section 2. (A) Exp. I, Figs. 4 and 5; (B) Exp. II, Figs. 4 and 5; (C) Exp. III, Figs. 8, 10 and 11; (D) Exp. IV, Fig. 9. For each group in each experiment the nature and duration of treatment, along with the number of cases (and see Tables 1 and 2), are given as indicated. The thick bars indicate the periods during which spontaneous firing was monitored for off-line computer analysis.

trajectories. Ontogenetic problems pose an exceptionally severe challenge since, as will become clear in the course of this review, potentially important effects can be obscured or missed altogether by the application of methods which are inherently too gross. Still further refinement is highly desirable, both in the selection of parameters to be measured and in the reduction of the enormous interindividual variance (among preparations of any given type) which plagues the field of experimental physiological ontogeny. Some modest suggestions for improvement in these respects are made in the course of reviewing the literature, and it is hoped that such a survey will stimulate others to find other ways in which progress can best be made (some preliminary reports on portions of these experiments have previously been published [134,503,724]).

2. Methodological approaches

2.1. Culture procedures

'Organotypic' slice cultures, made from 6-day-old rat occipital cortex, were prepared and maintained as described previously [25,30–38,583,587,588]. They were refreshed three times weekly, either with control growth medium or supplemented in such a way as to differentially reduce glutamatergic neurotransmission, thus modifying ongoing bioelectric activity (see below). Each experimental group comprised cultures taken from at least three different culture series, in order to minimize the chance that variations in tissues or experimental conditions from one run to the next could bias the results.

The following series of experiments were carried out, with corresponding control groups of untreated cultures run in parallel wherever appropriate (see Fig. 1 for overview).

2.1.1. Acute experiments

Exp. I: Either APV, DNQX or a mixture of both was added acutely to the recording medium at 1 week (7–10 days), 2 weeks (14–17 days) or 3 weeks (21–24 days) in vitro. SBA was recorded continuously for at least 2 h before returning the slices to the control medium. The acute effect of PTX addition was studied in some of the control cultures as well: whereas 10 μM was required in the younger cultures to induce maximally intensified spiking, 2 μM was found to be optimal in the 3-week-old group.

Exp. II: APV and/or DNQX were added to the growth medium 24 h prior to the recording session after 1-, 2- and 3-weeks of development in vitro. Dosages of APV and DNQX (20 and 50 μM, respectively) were chosen so as to just achieve maximal suppression of spontaneous spike discharges. The electrophysiological assay was first carried out in the overnight growth medium, and then in control recording medium.

2.1.2. Chronic experiments

Exp. III: Cultures were reared for 2 weeks either in control growth medium or in the presence of APV, with or without concomitant addition of DNQX. Recording was initiated an hour after transfer to control MEM. Most of these cultures were also recorded following the addition of APV or DNQX to the medium.

Exp. IV: Cultures were reared for 3 weeks, either in the APV/DNQX mixture or in APV alone. In this experiment, all cultures were placed in control growth medium

Table 1
Percentage of explants in each age and treatment group showing different mean levels of spontaneous firing (spikes/s) when measured in *control* recording medium or with PTX added acutely ('Low' firing is <0.1, 'medium' firing is 0.1–0.5, and 'high' firing is >0.5 spikes/s, averaged over the entire recording period. $*p < 0.05$ vis a vis the corresponding control group; $'p < 0.05$ vis a vis the chronic APV-treated group; $\wedge p < 0.05$ vis a vis the chronic APV + DNQX group. The difference between the 1- and 2-week control groups, and between the 2- and 3-week chronic-APV groups is also significant at the 5% confidence level)

Group	None	Low	Medium	High
1 week				
Control′	0.00	0.05	0.30	0.65
APV (24 h)*	0.30	0.20	0.30	0.20
APV (chronic)*	0.28	0.11	0.50	0.11
2 weeks				
Control′^	0.17	0.00	0.50	0.33
+ PTX (10 μM)	0.00	0.17	0.33	0.50
APV (24 h)	0.13	0.25	0.25	0.38
DNQX (24 h)	0.36	0.14	0.43	0.07
APV + DNQX (24 h)	0.10	0.00	0.70	0.20
APV (chronic)	0.17	0.17	0.28	0.39
DNQX (chronic)	0.25	0.08	0.21	0.46
APV + DNQX (chronic)′^	0.25	0.08	0.21	0.46
3 weeks				
Control′	0.00	0.00	0.60	0.40
+ PTX (2 μM)*^	0.00	0.00	0.00	1.00
APV (24 h)*′	0.33	0.06	0.17	0.44
APV (chronic)*^	0.00	0.00	0.17	0.83
APV + DNQX (chronic)′	0.21	0.07	0.29	0.43

Table 2
Percentage of explants in each age and treatment group showing different mean levels of spontaneous firing when measured either in APV for the indicated durations (with or without acute addition of PTX), or in DNQX(~)-containing medium ($*p < 0.05$ vis a vis the APV-24 h group; $'p < 0.05$ vis a vis the corresponding chronic APV group; $\wedge p < 0.05$ vis a vis the corresponding chronic APV + DNQX group; for definitions of firing groups, see Table 1)

Group	None	Low	Medium	High
1 week				
APV + PTX′	0.08	0.04	0.21	0.67
APV (24 h)′	0.09	0.14	0.41	0.36
APV (chronic)*	0.33	0.06	0.44	0.17
2 weeks				
APV (2 h)	0.09	0.33	0.58	0.00
APV (24 h)	0.11	0.22	0.39	0.28
~ DNQX (24 h)	0.43	0.43	0.14	0.00
APV (chronic)	0.18	0.18	0.41	0.23
APV + DNQX (chronic)	0.21	0.13	0.25	0.42
~ APV + DNQX (chronic)	0.23	0.23	0.23	0.32
3 weeks				
APV (2 h)	0.13	0.25	0.38	0.25
APV (24 h)	0.08	0.25	0.25	0.42
APV (chronic)^	0.00	0.17	0.50	0.33
APV + DNQX (chronic)*′	0.14	0.07	0.71	0.07
~ APV (chronic) *′	0.17	0.58	0.17	0.08
~ APV + DNQX (chronic)	0.14	0.43	0.43	0.00

for 24 h prior to being electrophysiologically monitored, first in control recording medium and then in APV or DNQX.

2.2. Electrophysiological assay

For recording, cultures were transferred to a recording chamber equipped with a stabilized DC warming circuit and a motorized piping system for the continuous circulation of aerated, bicarbonate buffered, MEM saturated with 10% CO_2. A small opening in the cover allowed electrodes to be positioned under visual control using motor-driven micromanipulators. Time *zero* for a given treatment, c.q. recording session, was defined as the moment that an air bubble introduced as a marker into the circulating medium was observed to reach the culture dish. Allowing ca. 1 h for adaptation, during which time the activity in the growth medium was checked, an analysis time of 15 min was routinely adhered to.

Saline-filled glass pipettes with a tip diameter of 2–3 μm were used for making extracellular recordings of spontaneous action potentials and local field potentials. The electrode was positioned within the relatively thick cell zone in the upper (i.e. pial) third of the explant ('layers II–IV': [25]). It was then moved slowly through the thickness of the slice until an optimal signal-to-noise ratio was obtained. Otherwise, after 3 min during which fewer than two 'slow-wave events' associated with 'bursts' of spikes (see Fig. 3) were observed, a nearby site was similarly probed. Only a single spike train was analyzed per explant, and a record was kept of the number of explants in each group which failed to show any spontaneous activity even after five sites had been examined (see Tables 1 and 2).

Electrical signals were high-pass filtered (200–5 kHz) and fed into a level detector set at a signal-to-noise ratio of ~2. Each spike triggered a square-wave pulse which was stored as a time stamp for off-line computer analysis. The raw signal was simultaneously recorded by an analog tape-recorder in order to subsequently check the reliability of the discrimination, as well as to routinely inspect the slow-waves and their association with the action potentials. Exhaustive examination of the first two dozen or so using an automated spike-sorting algorithm [329] indicated that the recorded action potential trains typically contained signals from one, two or, occasionally, three units.

2.3. Spike-train analysis

On the basis of our earlier experience with the quantification of time-series in similar material [149,281,551–554], temporal clustering of action potentials was chosen as the most likely parameter for revealing developmental and treatment effects on spontaneous spike trains. When all of the clearcut peaks in the interspike interval histograms (see Fig. 3) were plotted, it appeared that intervals longer than ~1.0 s optimally separated distinct slow-wave 'events'

Table 3
Quantitative aspects of field-wave associated spike-train clusters (network bursts: 1000 ms interspike interval criterion) for long-term treatments with glutamate receptor antagonists, measured in *control* medium (or with PTX added acutely) at different ages in vitro (for each spike train, c.q. explant, the burst *duration* is the mean length, in milliseconds, of all sequences of consecutive action potentials recorded at intervals shorter than 1000 ms; the burst *intensity* (spikes/s) is 1 + the reciprocal of the mean interspike interval, in seconds, within detected spike clusters. The burst *count* is the mean number of spikes per cluster, while the burst *variance* is the coefficient of variation over the calculated durations of all spike clusters in each train. All data were calculated for each explant separately, and are presented as the median (bold type) and 50-percentile range; *$p < 0.05$, **$p < 0.01$ vis a vis the respective *control* group; ′$p < 0.05$, ″$p < 0.01$ vis a vis the *chronic APV* group; ^$p < 0.05$, ^^$p < 0.01$ vis a vis the *chronic APV + DNQX* group)

Group	Duration	Intensity	Count	Variance
1 week				
Control	227–**682**–1345	9.9–**15.9**–33.0″	6–**10**–19′	2.0–2.8–3.3′
APV (24 h)	64–**104**–443*′	4.4–**20.6**–37.1″	3–**4**–5*	1.5–2.4–2.6
APV (chronic)	568–**993**–1487	3.6–**6.4**–8.6**	3–**4**–7*	1.5–2.0–2.4*
2 weeks				
Control	282–**467**–1705′	10.6–**14.2**–24.3′	7–**8**–18	1.2–2.0–2.2^
+ PTX (10 μM)	114–**229**–280**′^^	13.6–**14.1**–33.9′	4–**4**–4**′^^	1.5–1.8–2.0
APV (24 h)	132–**333**–347*″^^	9.4–**10.5**–29.3″	4–**4**–5*″	1.4–1.7–1.8*
DNQX (24 h)	185–**434**–607″^	13.3–**16.5**–21.3	4–**5**–8′^	1.3–2.3–2.6
APV + DNQX (24 h)	83–**176**–301**″^^	9.7–**17.5**–18.0	3–**3**–4**″^^	1.6–2.0–2.2^
APV (chronic)	1166–**1341**–1802*	5.6–**7.2**–9.3*	10–**12**–16	1.5–2.0–2.1^
DNQX (chronic)	175–**220**–619″^^	13.3–**13.5**–17.8	3–**5**–5*′^	1.8–2.1–2.3^
APV + DNQX (chronic)	455–**1270**–2078*	7.8–**11.3**–15.1′	9–**14**–23	1.1–1.6–1.9*′
3 weeks				
Control	546–**1268**–1693′^	9.7–**13.3**–15.9	8–**12**–23′	1.4–2.0–2.1′
+ PTX (2 μM)	565–**1655**–2195^	21.9–**23.3**–32.4**″^^	14–**32**–49*^^	1.0–1.2–1.7*
APV (24 h)	54–**162**–224**″	18.9–**20.2**–23.1**′	4–**4**–5**″	2.0–2.6–3.6*″
APV (chronic)	823–**2136**–3376*^	7.3–**13.4**–18.2	6–**27**–31*^	1.0–1.5–1.8*
APV + DNQX (chronic)	444–**700**–850*′	11.1–**12.6**–16.4	7–**9**–11′	1.6–1.6–2.0

from one another, whereas shorter intervals were mostly contained within such events. A value of 1000 ms was therefore chosen to be the cut-off point for calculating the incidence of slow-wave associated spike clusters in each culture, as well as the mean *duration* (and its *variance*), *intensity* of firing, and *spike count* (i.e. number of spikes) associated with such clusters (see Tables 3 and 4). Since such 'network-bursts' appear to be identical in this type of

Table 4
Quantification of network bursts (i.e. 1000 ms cluster criterion) in the presence of either DNQX (~) or APV—with or without acute addition of PTX—for the indicated time-span (~recorded in the presence of DNQX, otherwise in the presence of APV, acutely added to the medium. *$p < 0.05$, **$p < 0.01$ vis a vis the APV-24 h group; ′$p < 0.05$, ″$p < 0.01$ vis a vis the corresponding chronic APV group; ^$p < 0.05$, ^^$p < 0.01$ vis a vis the corresponding chronic APV + DNQX group; for definitions and conventions, see Table 3)

Group	Duration	Intensity	Count	Variance
1 week				
APV + PTX	14–**740**–1634*′	14.6–**22.6**–38.6′	8–**15**–22*	1.4–1.9–2.5
APV (24 h)	35–**122**–375′	21.1–**38.7**–52.8′	3–**6**–10	1.0–1.5–3.5
APV (chronic)	254–**844**–2254*	4.5–**6.9**–18.7*	3–**5**–14	1.4–1.5–2.6
2 weeks				
APV (2 h)	69–**77**–246′^	8.0–**15.1**–18.0*	2–**2**–3*″^^	1.5–2.2–2.5
APV (24 h)	38–**67**–200″^^	14.5–**47.5**–74.2′	4–**5**–6	1.9–2.9–3.4′^
~ DNQX (24 h)	1345–**1791**–2236*	13.0–**16.4**–19.8*	22–**23**–23*′^	1.2–1.3–1.4*
APV (chronic)	253–**886**–2221**	6.4–**7.8**–18.3*	5–**6**–9	1.4–1.5–2.0
APV + DNQX (chronic)	298–**611**–1437**	8.4–**12.0**–17.8*	4–**7**–14	1.3–1.8–2.2*
~ APV + DNQX (chronic)	369–**716**–1133	6.9–**10.1**–15.0	4–**6**–8	1.4–1.7–2.4*
3 weeks				
APV (2 h)	330–**417**–427′	3.3–**3.4**–13.5*″	2–**2**–3**″	1.0–1.1–1.5**′
APV (24 h)	74–**353**–822′^^	10.0–**42.0**–48.6^	5–7–11^	2.7–3.8–4.6′^
APV (chronic)	79–**183**–257*″^	22.9–**31.1**–43.6′^	4–**5**–12″	2.0–2.3–2.9*″^
APV + DNQX (chronic)	24–**39**–145**′^	30.1–**70.0**–190*′^	4–**4**–6*^	0.2–0.4–2.5*′
~ APV (chronic)	474–**587**–961*″	40.7–**44.5**–53.7′	23–**28**–31**″^^	0.5–0.6–1.0**″^
~ APV + DNQX (chronic)	53–**374**–1367^	15.8–**30.6**–46.7^	4–**8**–16″^	1.0–1.1–1.2**′

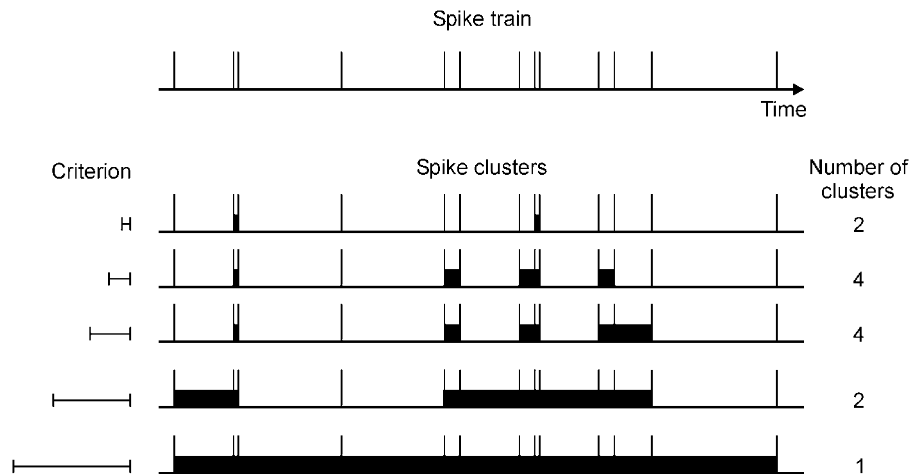

Fig. 2. Schematic outline of the burst-detection procedure. Demonstration, for a hypothetical spike train, how the *number* of clusters, their *durations*, and the spike counts will typically vary as a function of the cluster criterion (selected examples are indicated on the left of each trace).

preparation whether they occur spontaneously or are triggered by a single brief shock [143,162]; the calculated values for the burst parameters are pretty much the same as would have been obtained had we opted to experimentally evoke spike discharges rather than wait for them to appear on their own.

A computer analysis was also carried out using a sliding criterion for determining whether or not a given interspike

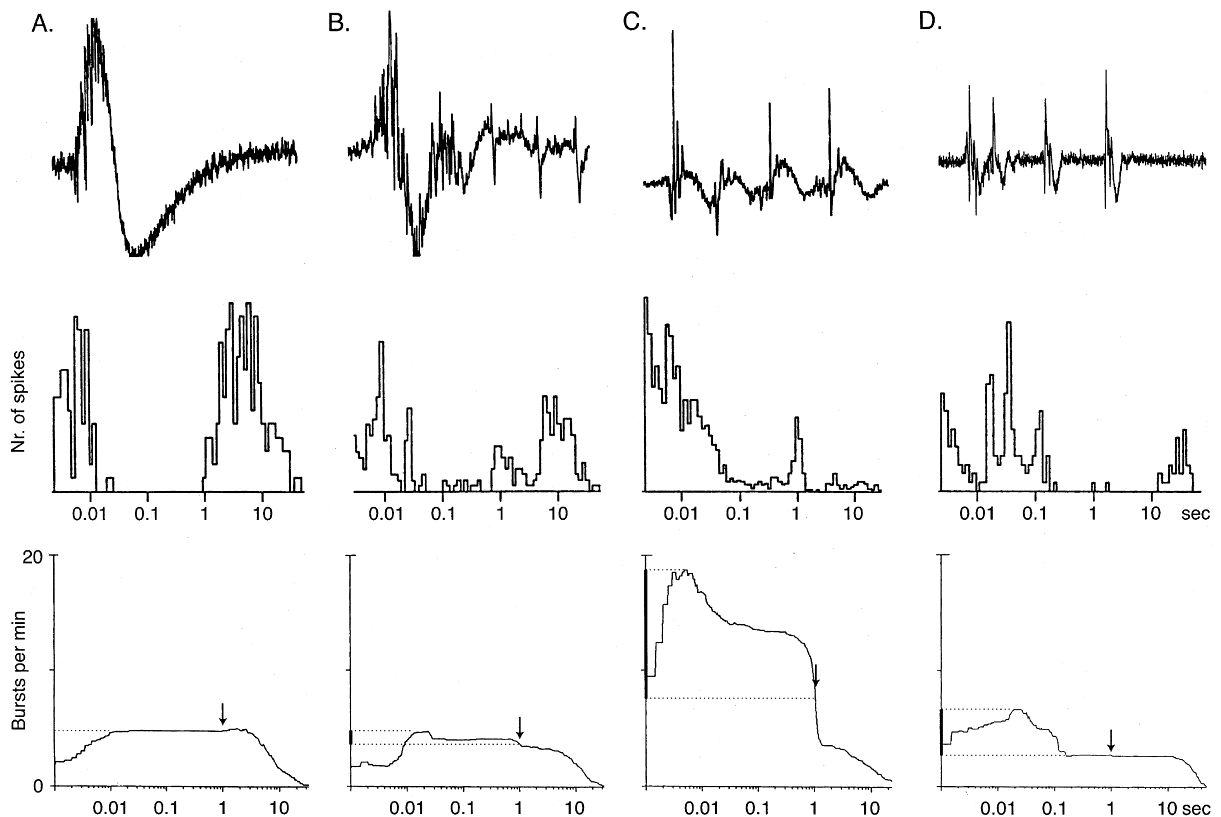

Fig. 3. Examples of field-potential associated neuronal *spike discharges* ((A–C) 100 ms sweeps; (D) 500 ms sweep); together with the corresponding interspike *interval histograms* (middle row: interspike intervals, in seconds, on the abscissa; relative spike incidence on the ordinate); and spike *cluster curves* (bottom row: bursts per minute on the ordinate, as a function of the cluster criterion on the abscissa). Dashed horizontal lines give the maximum incidence of spike clusters, regardless of the criterion value, as well as the incidence of 'network bursting events' (see (A); the arrows indicate the criterion value of 1000 ms used for defining a *network burst*). The difference between the two levels defines the incidence of 'mini-burst' after-discharges (darkened portions of the ordinate).

Table 5
Quantitative aspects of intra-burst clusters of action potentials (mini-bursts: 100 ms interspike interval criterion) for long-term treatments with glutamate receptor antagonists, when measured in *control* medium (or with PTX added acutely) at different ages in vitro; for definitions and conventions, see Table 3

Group	Duration	Intensity	Count	Variance
1 week				
Control	32–**52**–72″	70–**87**–117′	4–**5**–7″	1.0–1.3–1.8′
APV (24 h)	27–**33**–34**′	78–**88**–92′	3–**3**–4**″	1.0–1.2–1.2
APV (chronic)	16–**21**–24**	70–**119**–144*	2–**3**–3**	0.6–0.8–1.3*
2 weeks				
Control	27–**34**–71	63–**78**–107′	3–**4**–6	0.9–1.4–1.7
+ PTX (10 μM)	32–**40**–42	73–**76**–80′	3–**4**–4	1.0–1.3–1.4
APV (24 h)	31–**36**–37	69–**82**–100′	2–**3**–4*	0.6–1.0–1.2
DNQX (24 h)	31–**43**–65	55–**61**–86	3–**3**–5	1.0–1.2–1.5
DNQX + APV (24 h)	24–**35**–44	48–**58**–65*″	2–**3**–3*^	0.8–1.0–1.1
APV (chronic)	10–**22**–48	78–**126**–144*^	3–**3**–4	0.9–1.2–1.7
DNQX (chronic)	13–**14**–29	99–**144**–174*^^	3–**4**–4	0.6–0.8–0.9*′^
APV + DNQX (chronic)	35–**58**–70′	57–**70**–78′	3–**4**–6	1.0–1.2–1.8
3 weeks				
Control	29–**63**–72	57–**73**–147	4–**4**–5	0.9–1.2–1.6
+ PTX (2 μM)	84–**93**–112**″^^	67–**70**–76^	7–**8**–9**″^	1.1–1.2–1.3
APV (24 h)	17–**20**–32**′^	95–**151**–161*″^	4–**4**–5	0.6–0.7–0.8**″^
APV (chronic)	38–**46**–59	57–**72**–78^	3–**5**–5	1.1–1.2–1.7
APV + DNQX (chronic)	36–**52**–66	75–**84**–118′	4–**5**–6	0.9–0.9–1.9

interval should be regarded as belonging to a 'cluster' (see Figs. 2 and 3 [118]; for a discussion of the limitations of this approach, see Ref. [347]). Initially low (zero in the case of single unit recordings), the number of clusters in a given record typically increases to a maximum as more and more intervals fall within the criterion value (Fig. 2). In the absence of mini-bursts within each network-burst (see below), the curve will exhibit a plateau extending into the >1.0 s range, viz. the cut-off value at which a given interspike interval by definition heralds the termination of a slow-wave associated 'event'. At some point (depending on the intervals between successive network-bursts) the curve will begin to decline, with a slope determined by the degree of temporal regularity of these slow-wave/spike events (Fig. 3).

Clusters of spikes separated by intervals shorter than 1000 ms were defined as mini-bursts (MB) contained within a slow-wave/burst event (see Fig. 3). When such a tendency exists for spiking to be clustered *during* these network-bursts, a decline in the cluster-incidence curve from its maximum to a lower plateau value will already begin prior to the 1000 ms cut-off point, and from the position and amplitude of such transitions the incidence of mini-bursts (MBs) can be determined, and the intervals between them estimated (Fig. 3). Since treatment and developmental effects turned out in these experiments to be in the same direction for all categories of MBs, they were pooled for statistical analysis. The peak value of the 'cluster curve' (minus the incidence of network-burst events) in itself thus sufficed to calculate the overall incidence of MBs in a given spike train.

Interspike interval histogram analysis revealed that, in cases where the distribution of intervals <1.0 s was polymodal (see Fig. 2), the trough which best separated the relatively short intervals from the longer ones was usually located close to the 100 ms point on the abscissa. This value was therefore selected as the cluster criterion for characterizing the temporal fine structure of spiking during slow-wave associated network burst events. To this end, the *duration* (in milliseconds), the *intensity* of firing (in spikes per second) and the spike *count* (spikes per cluster) were calculated for each spike train, with interspike intervals of <100 ms serving to define these mini-bursts (see Tables 5 and 6). It should again be emphasized that the values thus calculated are probably the same as would have been obtained, had electrically evoked rather than spontaneous burst discharges been analyzed [141,162].

In many cases a slow fluctuation in firing level was evident in the time-histogram, with a period of one or several minutes per cycle (see Fig. 14). Successive cycle lengths were estimated on the basis of time histograms using consecutive 1 s bins, and the mean value was calculated for each of the preparations showing such periodicity. In addition, the coefficient of variation (CV 60″) was calculated for the number of spikes counted in successive 60 s time-bins. This procedure smoothens out all fluctuations in spontaneous firing which are faster than 1 min/cycle, and was shown in earlier in vitro studies to be a particularly sensitive indicator both for developmental changes in SBA [281] and for pharmacological treatment effects [551].

2.4. Statistical testing

Only explants showing a mean firing rate (MFR) of at least 0.1 spikes/s were included in the statistical procedures (see Tables 1 and 2 for the proportions of inactive and

Table 6
Quantification of spike-train mini-bursts (i.e. 100 ms cluster criterion) for recordings made in the presence of either DNQX (~) or APV—with or without acute addition of PTX—for the indicated time-span (~recorded in the presence of DNQX, acutely added to the medium; for conventions and definitions see Table 4)

Group	Duration	Intensity	Count	Variance
1 week				
PTX + APV	40–**76**–93*″	67–**116**–133	6–**7**–10*″	0.5–0.7–1.0′
APV (24 h)	15–**27**–75	60–**85**–173	3–**4**–5′	0.6–0.9–1.3′
APV (chronic)	14–**23**–32	69–**84**–134	2–**3**–4*	1.0–1.3–1.5*
2 weeks				
APV (2 h)	16–**31**–33	33–**54**–103*	2–**2**–3*′	0.4–0.5–0.7′^
APV (24 h)	10–**14**–20^^	101–**226**–334′^^	3–**4**–4	0.2–0.3–0.5″^^
~ DNQX (24 h)	48–**79**–111*	111–**158**–205	6–**8**–9	0.8–1.2–1.5*
APV (chronic)	16–**20**–25^^	117–**135**–151*^^	3–**3**–4	0.6–0.9–1.2**
APV + DNQX (chronic)	26–**47**–64**″	58–**81**–92**″	3–**5**–5	0.7–1.0–1.2**
~ APV + DNQX (chronic)	24–**38**–70	51–**94**–117	3–**4**–5	0.7–1.2–1.5
3 weeks				
APV (2 h)	12–**15**–16*′^	136–**159**–245*′^	2–**2**–3*′^	0.3–0.3–0.4*
APV (24 h)	33–**40**–41^	101–**109**–147^	3–**5**–7	0.4–0.9–1.1^
APV (chronic)	18–**30**–49″	80–**111**–151^	4–**4**–5	0.4–0.4–0.9
APV + DNQX (chronic)	18–**19**–28*	124–**169**–226*′	4–**4**–6	0.2–0.4–0.5*^^
~ APV (chronic)	71–**385**–558**″^	65–**71**–77**′^^	21–**26**–29**″^^	0.6–0.7–1.1
~ APV + DNQX (chronic)	41–**78**–159*′	45–**48**–54″	4–**6**–9″	0.9–0.9–1.0^^

weakly active recording sites in each group). If a multivariate analysis (Benferroni, Kruskal–Wallis or χ^2 test) revealed the existence of a significant difference at the 5% confidence level, pairwise group comparisons were carried out using the Mann–Whitney U, χ^2, or Fischer 'exact-p' tests (two-tailed except when the difference between two groups was in the direction hypothesized on the basis of pilot studies or earlier published experiments: [149,551–554]). Acute treatment effects within groups were evaluated either by means of the Wilcoxon matched-pairs signed-ranks test or the Sign test, with $p < 0.05$ considered statistically significant.

3. Experimental findings

3.1. Short-term effects of glutamate receptor blockade on spontaneous firing patterns

Addition of either APV or DNQX to the growth medium quickly caused a strong reduction in spontaneous firing when administered separately in control cultures at all ages, but both of these pharmaca in combination were required for a total suppression (or very near total in a few cases) of extracellularly measured action potentials. This effect persisted from one refreshment to the next (data not shown, but see Fig. 4: APV + DNQX-24 h) and could be maintained for above 3 weeks.

DNQX alone, both acutely and after overnight exposure, induced infrequent trains of spikes (see Fig. 4) which, however, were greatly prolonged and with a corresponding augmentation of the mean number of spikes in each network-burst event (Table 4; [551]). This intensification of intra-burst firing above control values was also evident for the mini-bursts (Table 6). The overall firing levels were nevertheless drastically reduced in the presence of DNQX, and almost half of such preparations failed to show any SBA at all (Fig. 1(B)). Removal of DNQX from the recording medium, even after 24 h, led to firing levels and patterns which did not differ noticeably from the controls (Fig. 4; Tables 1, 3 and 5).

APV had an especially striking acute effect on spontaneous firing in 2-week-old cultures: after an initially strong suppression (Fig. 4), SBA levels began to rise again after 0.5 h or more of exposure, with the time of onset and the extent of recovery varying widely from explant to explant (averaging ca. 50% by 2 h after 'time-zero': Fig. 4; also see Tables 2, 4 and 6). Return to control medium quickly restored SBA levels to normal (Fig. 4; Table 1). One-week-old cultures reacted to APV in a similar fashion (data not shown) whereas the 3-week-old group showed little or no recovery of activity levels even after 24 h (Fig. 5).

After overnight treatment, 1- and 2-week-old cultures showed significantly enhanced mini-burst and overall firing levels with APV still present in the recording medium, despite a slightly reduced incidence of network bursts (see Fig. 4: '24 h' group). In sharp contrast to control cultures, however, this activity proved to be resistant not only to fresh APV (data not shown) but also to the alternative NMDA receptor blocking agent, MK-801 (Fig. 4). Three-week-old cultures, on the other hand, were strongly depressed after 24 h in APV (Fig. 5; Table 2). Only in this latter age group did a return to normal MEM (i.e. the control recording medium) lead to a significant increase in activity levels (Fig. 5), although the percentage of silent cultures remained abnormally high (Table 1).

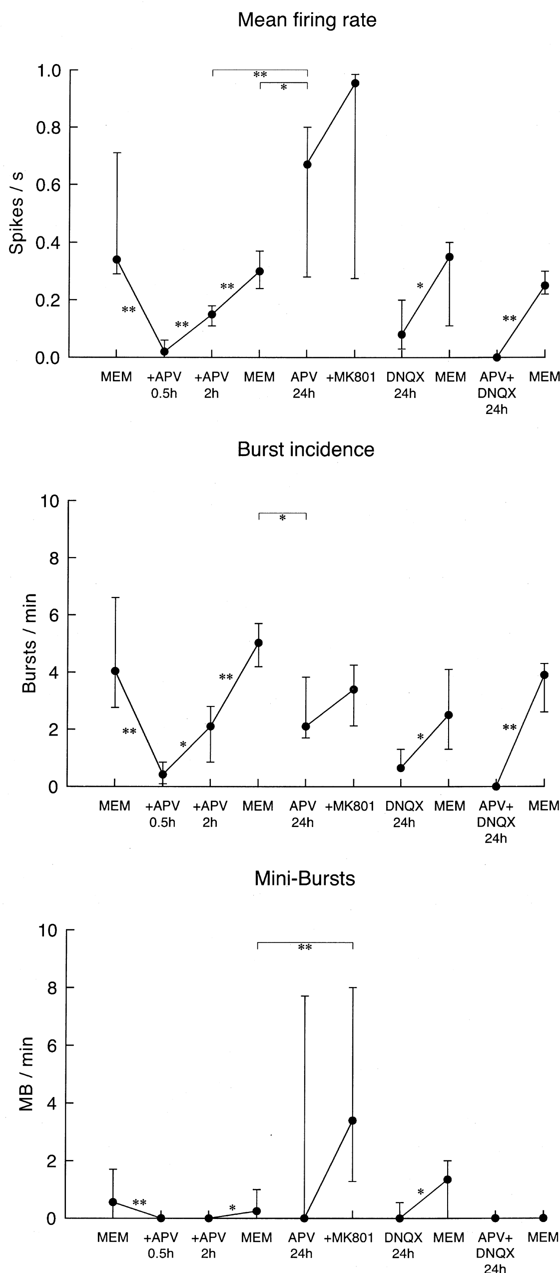

Fig. 4. Effects of NMDA and/or non-NMDA receptor blockade on spontaneous action potential generation in 2-week-old cultures, as a function of time after addition of APV (left-hand curves: MEM…MEM), and 24 h after exposure to APV and/or DNQX; $*p < 0.05$, $**p < 0.01$.

Further analysis revealed that spontaneous network bursts in cultures of all ages had become considerably shortened by 24 h exposure to APV (Table 4) and that this held true even when SBA was subsequently monitored in APV-free medium (Table 3). The mean number of action potentials per burst was correspondingly reduced in all groups (see Tables 3 and 4: *spike count*), leaving the *intensity* of firing within bursts largely unaffected. Mini-bursts were affected by overnight APV treatment in much the same way as the overall network-bursts, albeit to a lesser degree (see Tables 5 and 6). Interestingly, when 2- but not 3-week-old cultures were returned to control medium they mimicked acutely PTX-treated explants (see below) in that their network-bursts were shorter (Fig. 2) and exhibited a significantly heightened degree of stereotypy (data not shown).

Spontaneously active explants exposed acutely to PTX at 2 weeks in vitro showed a consistent tendency to fire in relatively short-lasting network bursts (Table 3); the intensity of the brief initial discharges was unaffected, however (Table 5). At 3 weeks in vitro, in contrast, the initial discharges (mini-bursts) became significantly prolonged following acute PTX treatment (Table 5) while the overall intensity of clustered spiking episodes increased greatly (Table 3). Neither of these changes was sufficient to be significantly reflected in the distribution of MFRs (Fig. 6; [553]), despite the fact that PTX increased the proportion of rapidly firing units (Table 1). A singular effect of acute exposure to PTX at 3 but not at 2 weeks in vitro was an unusual degree of consistency in the durations of consecutive network bursts (Table 3).

Some of the 1-week-old control cultures were exposed to PTX in the presence of APV shortly after the acute effect of the latter had been recorded; within minutes the PTX treatment had restored a number of activity parameters to the level observed prior to APV administration (Fig. 7). A tendency for SBA to take the form of relatively intense but short-lasting clusters of action potentials was nevertheless visible: spike clusters under these conditions showed a trend towards greater mean durations and intensity of firing (Tables 3 and 4), especially with respect to the mini-bursts (Tables 5 and 6). At all ages, acute PTX treatment of cultures which had been exposed for 24 h to APV led to a still further enhancement of SBA (data not shown).

3.2. Spontaneous firing following chronic total blockade of glutamate receptors

An approximately twofold increase above control levels can be seen in the incidence of mini-bursts per network-burst in the 2-week-old APV + DNQX treated group after return to normal medium (Fig. 8). The incidence of network bursts themselves in these cultures showed no difference whatsoever, but they tended to last considerably longer than in the control group and to contain a larger number of action potentials (Table 3), a fact which presumably accounts for the trend towards higher overall firing rates which can be seen in Fig. 8. The individual mini-bursts making up each network burst appeared to be perfectly normal in all respects (Table 5), there simply being a lot more of them in the experimental group (see Fig. 8). In both treated and untreated explants, interindividual differences in spike cluster *durations* were correlated negatively with the overall *intensity* of firing during the bursts, whether the

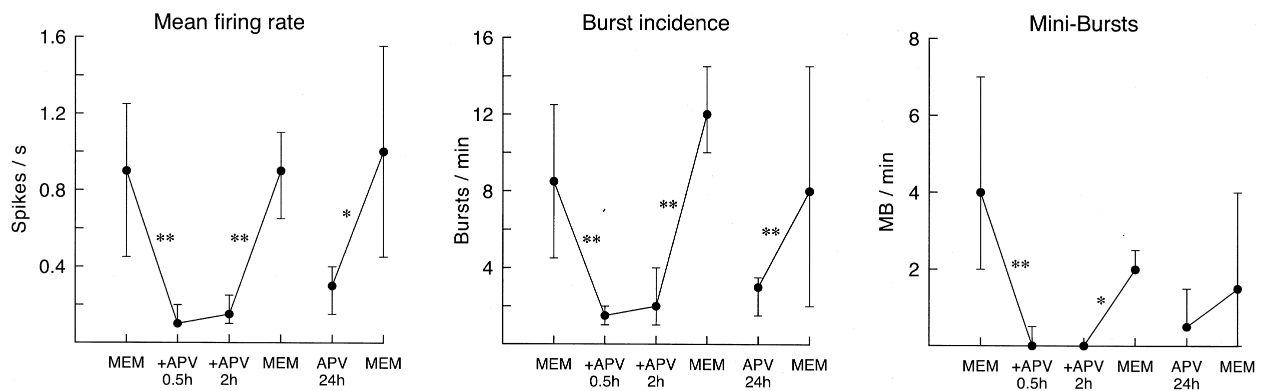

Fig. 5. Effects of NMDA receptor blockade on firing patterns in 3-week-old cultures at different times following application of APV to the medium; $*p < 0.05$, $**p < 0.025$.

interspike interval criterion used to define them was 100 or 1000 ms ($p < 0.01$; data not shown).

The physiological effects of total glutamatergic receptor blockade were much more severe after 3 than after 2 weeks of chronic exposure, despite the fact that the older explants had been allowed to recover for 24 h in normal growth medium prior to being recorded. Thus, 3-week-old cultures which had been treated continuously with APV + DNQX showed mean discharge rates which were twice as high as in control cultures, along with an equally enhanced incidence of mini-bursts (Fig. 9). Mini-bursts were fully normal in all respects (Table 5) but the network burst events (surprisingly, considering that they occurred no more frequently than in the control group: see Fig. 9) were considerably *shorter* than normal (Table 3). In addition, a trend could be seen towards a higher incidence of inactive or low firing recording sites (Table 1). As at 2 weeks in vitro, in both experimental and control cultures, interindividual differences in the *durations* of spontaneously occurring spike bursts were negatively correlated with *intensity* of firing whether an interspike interval criterion of 100 or of 1000 ms had been used ($p < 0.01$; data not shown).

SBA levels measured at 3 weeks in vitro in the presence of DNQX were significantly higher in the chronic APV + DNQX group than in control cultures, although the differences were not as large as at 2 weeks (data not shown). Network bursts tended to be shorter but more intense in the chronically treated cultures under these conditions of acute non-NMDA receptor blockade, so that the mean number of spikes per burst was practically identical in the two groups (Tables 3 and 4). Mini-bursts, in contrast, were relatively short but intense in the *control* cultures under such conditions (Tables 5 and 6). The incidence of weak or inactive sites was, not unexpectedly, much higher when DNQX was present in the medium than when it was not (Tables 1 and 2: cf. chronic APV + DNQX groups).

SBA levels measured at 3 weeks in vitro in the presence of APV were found to be significantly higher in the experimental than in the control group; indeed, the overall firing levels in chronically APV + DNQX treated cultures under

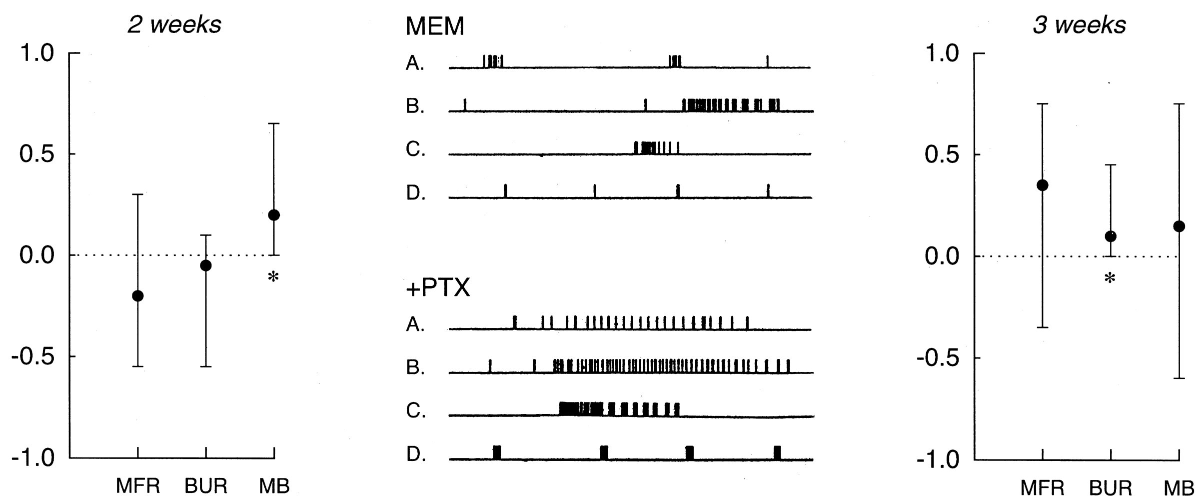

Fig. 6. Acute effects of PTX on quantitative aspects of SBA in 2- and 3-week-old cultures: MFR, mean firing rate; BUR, burst events; MB, mini-bursts; $*p < 0.025$; the spike traces (10 s sweeps) show the changes in firing patterns observed in four 3-week-old cultures (A–D).

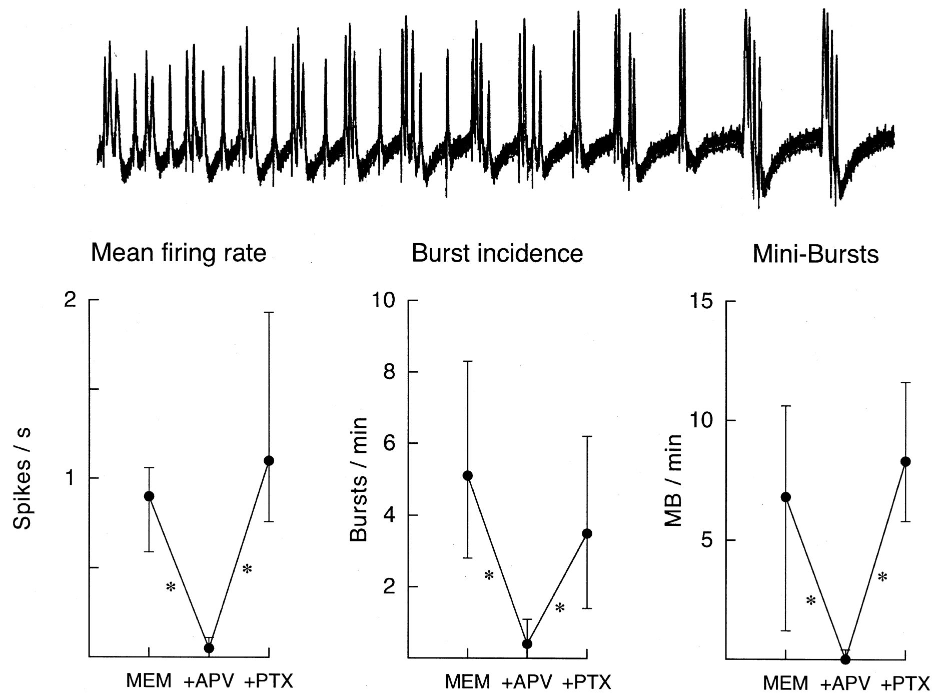

Fig. 7. Acute effects of NMDA receptor blockade (MEM + APV) on spontaneous firing in 1-week-old cultures, and its reversal by GABAergic disinhibition; $*p < 0.01$. (Above: a 1 min sweep showing an especially prolonged train of action potentials following PTX addition to the recording medium).

conditions of acute NMDA receptor blockade were indistinguishable from those in control cultures recorded in normal medium (data not shown). Network burst events were much shorter-lasting but intense, however, in experimental than in control cultures (also when the latter were monitored in the presence of APV after exposure for 24 h: Table 4). A similar shift towards shorter but more intense spike clusters was noted for mini-bursts in the chronically silenced group (Table 6). Acute exposure to APV had little or no effect in this group on the proportions of recording sites showing low firing rates or no activity at all (cf. Tables 1 and 2), this in contrast to the noticeable shift towards weaker SBA in cultures exposed to APV for only 24 h.

3.3. Spontaneous firing following chronic selective glutamate receptor blockade

Chronic treatment with *DNQX* for 1–2 weeks led consistently to the virtually complete disappearance of measurable SBA until the cultures were transferred to normal medium. Although network bursts soon occurred at approximately the same frequency as in control cultures, they were almost completely lacking in mini-bursts with a concomitant strong depression of MFRs (Fig. 8; Tables 1 and 3). Mini-bursts were shorter but more intense than in the controls, so that the onset portion of the overall network discharge did not contribute to the observed reduction in spontaneous firing (Table 5).

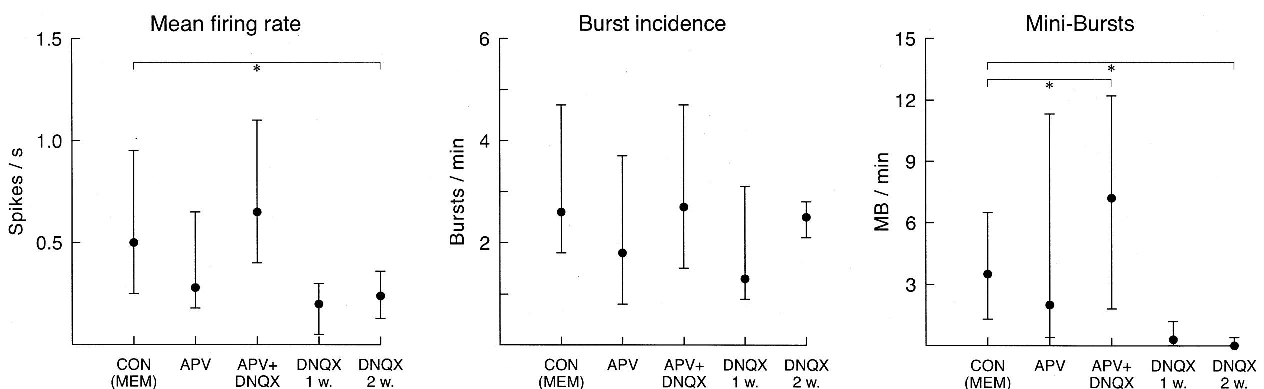

Fig. 8. Spontaneous firing patterns at 2 weeks in vitro, as monitored in MEM ca. 1 h after the termination of chronic exposure to APV, DNQX or a combination of both; $*p < 0.025$.

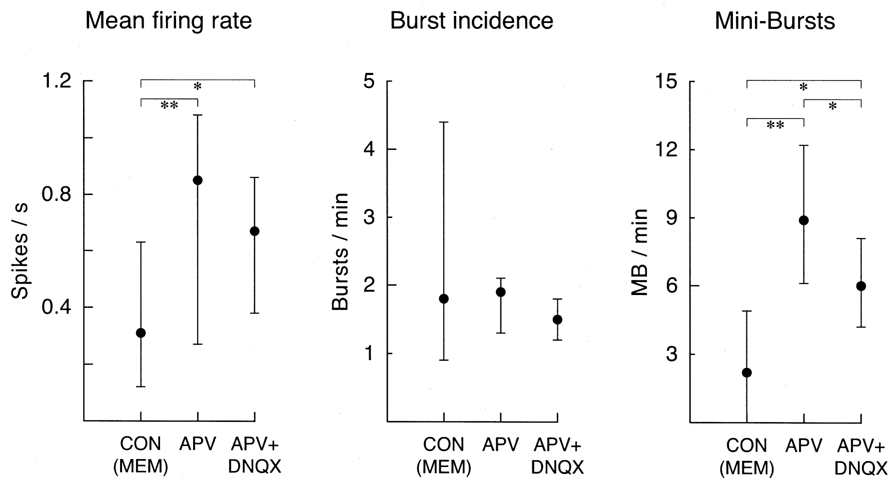

Fig. 9. Spontaneous spike train patterns at 3 weeks in vitro, as monitored 24 h after return to control growth medium, in chronically APV or APV + DNQX treated cultures; $*p < 0.025$; $**p < 0.01$ as compared with controls.

After 2 weeks of continuous *APV* treatment, on the other hand, no abnormalities in burst incidence or overall firing levels were evident when the cultures were monitored in control medium (Fig. 8; Table 1). Applying the 1000 ms spike cluster criterion, however, the network-bursts measured in this group proved to be longer (but less intense) than in the controls (Table 3). With a cluster criterion of 100 ms, a slight shortening of mini-burst durations together with a concomitant enhancement of mean spike-intensities was the only noticeable effect (Table 5).

After 3 weeks in vitro, chronically APV-treated explants showed enhanced spontaneous firing rates in control recording medium, even in comparison with the chronically silenced, i.e. APV + DNQX-treated cultures (Fig. 9; Table 1). Network bursts tended to be enormously prolonged and to contain more action potentials than in the controls (Table 3). The mini-bursts comprising each of the network discharges appeared to be perfectly normal, however (see Table 5), there simply being many more of such mini-bursts than in control explants (Fig. 9).

Recordings made shortly after the addition of APV to the medium revealed that chronic NMDA receptor blockade had indeed caused considerable changes in the developing cortical networks already by 2 weeks in vitro. Thus, cultures treated chronically with APV proved to be highly resistant to APV added acutely to the recording medium: whereas SBA in control cultures was virtually abolished by such treatment, the chronic-APV group was scarcely affected (Fig. 10), even less so, in fact, than in cultures which had been totally silenced for 2 weeks by combined APV + DNQX treatment. Similarly, mini-burst characteristics in the chronic-APV group showed little or no change when APV was added acutely to the recording medium (cf. Tables 5 and 6).

Firing levels in 2-week-old chronic-APV cultures were depressed to a much greater extent by acute DNQX (Fig. 11)

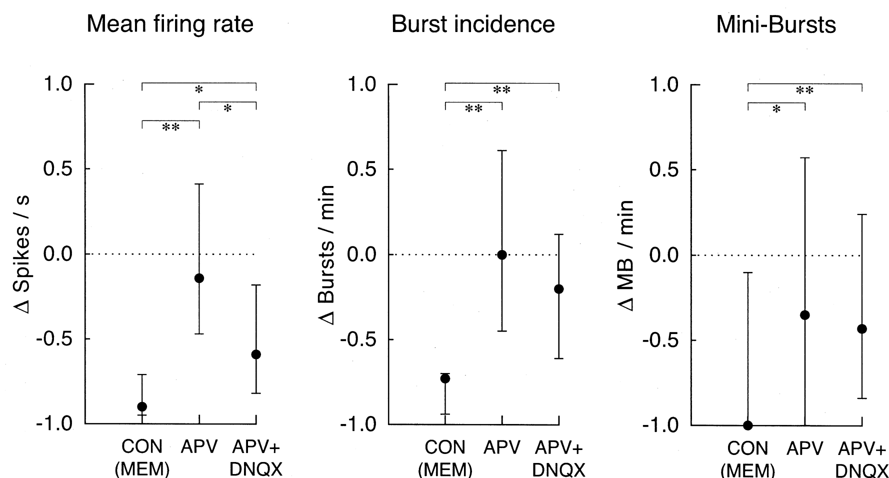

Fig. 10. Effects of NMDA receptor blockade (i.e. acute exposure to APV) at 2 weeks in vitro on spontaneous firing in APV- and APV + DNQX *chronically* treated, as compared with control cultures. Proportional changes from the baseline level are indicated on the ordinate; $*p < 0.025$, $**p < 0.01$.

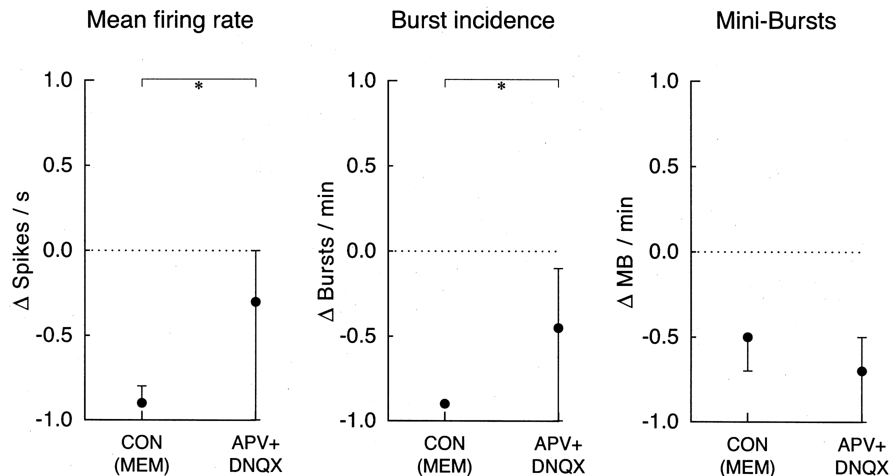

Fig. 11. Effects of non-NMDA receptor blockade (i.e. acute exposure to DNQX) at 2 weeks in vitro on spontaneous firing in cultures chronically exposed to APV + DNQX, in comparison with untreated control cultures. Proportional changes are indicated on the ordinate; $*p < 0.025$, $**p < 0.01$.

than by acute APV exposure, with the vast majority of recording sites showing only weak activity or none at all in the former case (Table 2). As in acutely treated cultures, however, such bursts which continued to occur in the presence of DNQX, took the form of prolonged, high intensity discharges containing an extremely large number of action potentials (Tables 4 and 6). In striking contrast, SBA in the chronic-APV group when re-exposed to APV consisted of abnormally brief network bursts, containing relatively few spikes despite their high intensity (Table 4). Spontaneous firing during mini-bursts, in contrast, was only slightly enhanced (Table 6).

A schematic overview of the most characteristic burst structures in diverse experimental groups is provided in Fig. 12.

3.4. Ontogeny of slow fluctuations in spontaneous firing levels

Pronounced ‘slow’ fluctuations (viz. longer than 0.7 min/cycle) in MFRs were observed in about 50% of the 1-week-old control cultures, as well as in older cultures that had been acutely disinhibited by addition of PTX to the recording medium (Fig. 13). For both groups, the modal value for SBA periodicity was close to 1 min, and in all explants fell within 2.5 min as an upper limit.

Both 2- and 3-week-old control cultures displayed a higher proportion of cases showing such slow SBA rhythms, and with a longer mean period on the whole, than did either untreated 1-week-old or acutely PTX exposed older explants (Fig. 13). This difference was due to the existence in the older age control groups of a discrete subclass of recording sites at which unusually slow fluctuations in firing level (3–5 min/cycle) were encountered. Such fluctuations most likely account for the progressive slight increase with age seen in the CV 60″ parameter (Table 7).

At both 2 and 3 weeks in vitro, chronically APV or APV + DNQX treated cultures showed only a few instances of the longer activity cycles (Fig. 13), even though the incidence of such ‘minute-order’ rhythmicity was much the same as in untreated control explants. As a result, minute-to-minute fluctuations in spike discharge rates were observed to be considerably less extreme in both the experimental groups than in the controls, with this effect being greater at 3 than at 2 weeks (Table 7). The regularity of successive *cycle lengths*, however, was comparable in all age and treatment groups: coefficient of variation = 20–40% (data not shown).

Table 7
Minute-to-minute fluctuations in spontaneous firing rates (coefficient of variation calculated over the number of action potentials in consecutive 60 s time-bins) when measured in control recording medium, with or without acutely added picrotoxin ($*p < 0.05$, $**p < 0.01$ vis a vis the respective *control* group; $'p < 0.05$, $''p < 0.01$ vis a vis the *chronic APV* group; $^\wedge p < 0.05$, $^{\wedge\wedge}p < 0.01$ vis a vis the *chronic APV + DNQX* group)

Group	1 week	2 weeks	3 weeks
Control	0.7–**1.1**–1.8	0.8–**1.2**–2.4′^	1.0–**1.3**–2.1″^^
Control + picrotoxin	–	0.4–**0.4**–0.5**′^	0.5–**0.5**–1.0*
APV (24 h)	0.7–**0.7**–0.8*	0.6–**0.6**–0.9*	0.6–**0.7**–1.0**
DNQX (24 h)	–	0.7–**1.2**–2.0	–
DNQX + APV (24 h)	–	0.4–**0.4**–0.5**′^	–
APV (chronic)	0.7–**0.8**–1.1	0.6–**0.9**–1.0*	0.5–**0.7**–1.0**
APV + DNQX (chronic)	–	0.5–**0.9**–1.2*	0.7–**0.8**–1.1*

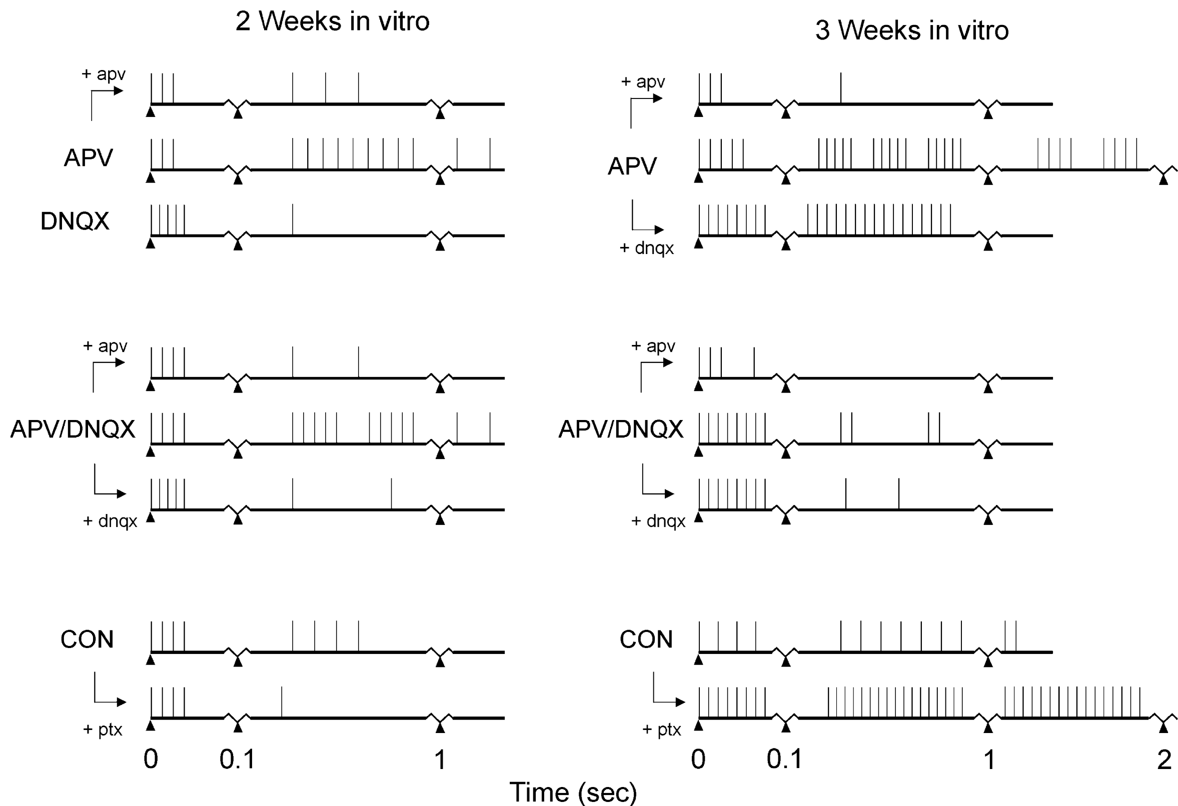

Fig. 12. Schematic overview of 'typical' bursting patterns in cultures treated chronically with glutamate receptor blockers (APV and/or DNQX) or untreated as controls. Recordings were made in normal MEM except where acute addition of *apv*, *dnqx* or *ptx* is indicated by (+). Filled triangles under each sweep indicate the **zero**, **100**, **1000** and (where necessary) **2000** ms time points. The initial spike cluster characteristics were derived from tables (see Section 4) giving the mean burst *durations* and *counts*, calculated using an interspike interval criterion of 100 ms. The remainder of the average network burst characteristic for each condition was calculated (leaving a spike-free interval of >100 ms, but note the differences in scale between the successive triangles) from the 1000 ms cluster criterion tables, together with the mean number of after-discharges per group (no attempt has been made to represent quantitative trends which can be expected to exist within or between the mini-bursts contributing to the recorded spike discharges).

Similar 'circa-minutial' alternation of active and quiescent periods was also observed in the contractions of dissociated (rodent) heart cells cultured for several days in vitro (Fig. 14). Simultaneous recordings from neurons cultured on a multi-electrode plate for up to ~3 weeks [725,750] have indicated that, when present, such rhythms are often free-running with respect to one another even when the recording sites are very close to one another (Fig. 15).

4. Summary of the present results

The development of spontaneous single and multiple unit action potential trains, and their dependence upon glutamatergic synaptic transmission, has been studied in organotypic slice cultures ('explants') made from early post-natal rat neocortex tissue. Spontaneous activity was already well developed by 1 week in vitro and, at all ages, showed an extremely wide variety of firing patterns and levels from one explant, c.q. recording site, to the next. Clearcut tendencies were observed for action potentials to cluster over a broad range of time-scales, varying from a fraction of a second to several minutes. A newly devised method based upon systematically varying the criterion for defining bursts was applied, aided by analysis of the corresponding interspike interval histograms, in order to quantify the incidence of such patterns of SBA. *Network-burst events* were able to be usefully defined as clusters of action potentials separated by an interval of 1 s or longer, with more rapid spike clustering being defined as mini-bursts belonging to a single event. Fine details of burst structure were quantified by calculating the burst *length* (in milliseconds), *intensity* (spikes per second during the average burst) and *count* (spikes per burst) for each recorded spike train (one per explant), using spike clustering criteria of 100 ms (for the mini-bursts) as well as 1000 ms for the network-bursts.

NMDA receptor blockade (acute APV treatment) drastically reduced SBA at all ages but, in cultures up to 2 weeks in vitro, the activity recovered gradually in level—but not in pattern—over a period of several hours. SBA under such conditions was characterized by abnormally short and intense bursts of firing, and was now completely dependent on non-NMDA receptor activation (i.e. they were insensitive to both APV and MK801). Disinhibition by means of PTX neutralized the acute effect of APV, leading to a firing pattern similar to that seen both in

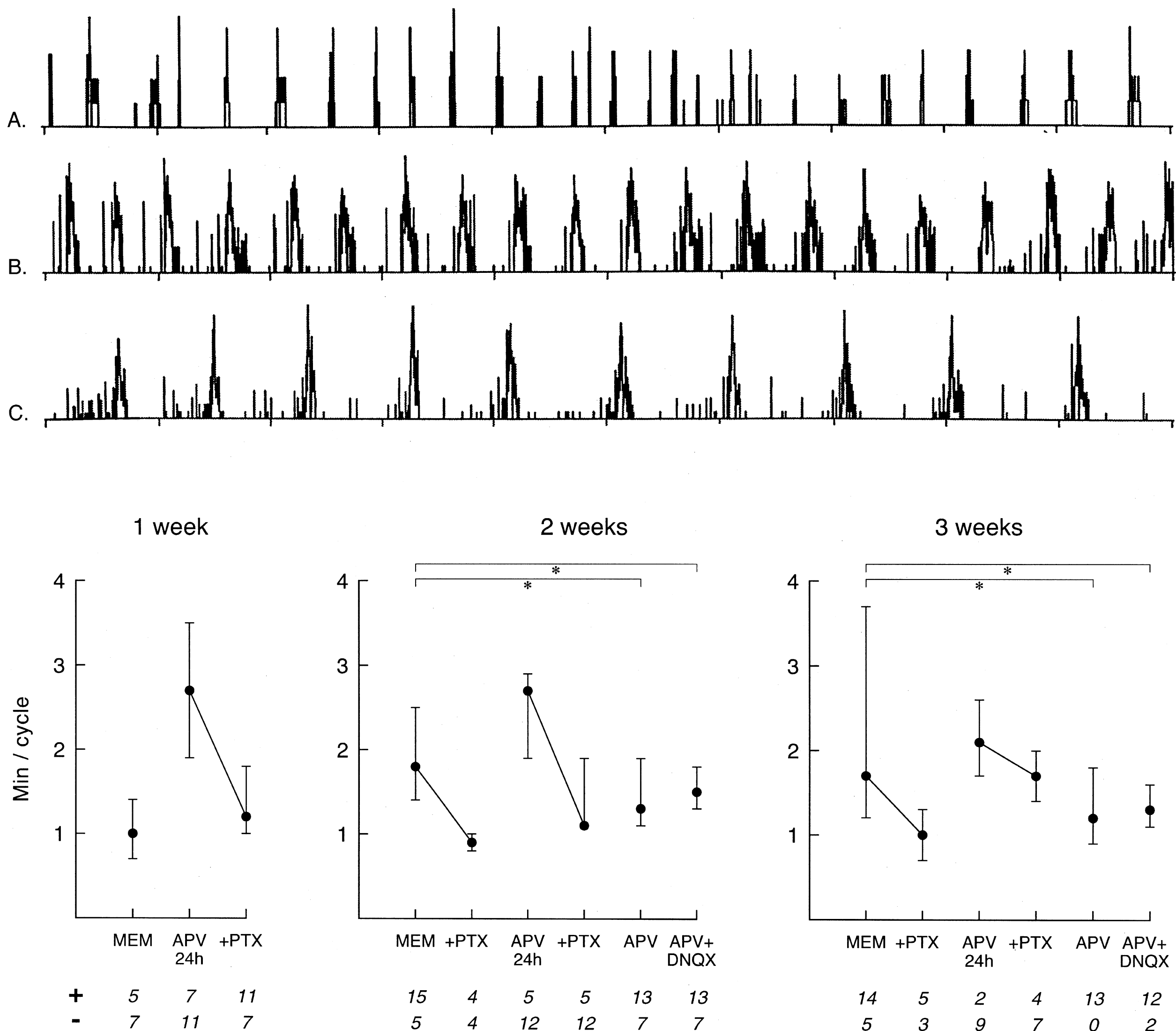

Fig. 13. Slow rhythms of firing in cultured cortical explants: three examples (A–C) illustrating the range of periodicities encountered. Each trace represents a continuous half-hour recording (3 min/div.). *Under*: modal and 50-percentile values are given for control (MEM) and short-term APV treated (APV-24 h) cultures at 1, 2 and 3 weeks in vitro; acute effects of picrotoxin (+PTX) in 1-, 2- and 3-week-old cultures; and the effects of chronic blockade of NMDA and/or non-NMDA receptors (APV and APV + DNQX, respectively) at 1-, 2- and 3-weeks in vitro; $*p < 0.05$.

control cultures treated acutely with PTX and in cultures monitored after 24 h in APV. In the latter group of explants (in which SBA had returned to control levels) PTX induced still more frequent and intense burst firing. Non-NMDA receptor blockade (acute DNQX treatment) transformed SBA at all ages into a pattern of infrequent, but greatly prolonged spike bursts. No recovery was seen even after 24 h. A 'cocktail' of DNQX plus APV succeeded in almost completely suppressing spiking for as long as this treatment was continued, but with return of qualitatively normal activity within minutes after bathing the cultures in control medium.

Chronic exposure to APV + DNQX for 3 weeks led to hyper-excitability of the treated cultures when monitored after 24 h in control medium: the incidence of network bursting events was not noticeably affected, but each cluster of spontaneous action potentials was composed of many more mini-bursts (i.e. high frequency spike clusters) than in untreated control cultures. Since such network bursts were nevertheless relatively short-lasting, while the overall firing rates were much higher in experimental than in control cultures, it can be deduced that the level of firing in between bursts must also have been considerably higher than in the controls. Cultures treated chronically with only APV added to the growth medium showed even more extreme deviations from control values than did the APV + DNQX cultures at this age, despite the fact that abundant SBA had been present throughout the treatment period in the former group (albeit in a somewhat 'paroxysmal' pattern). Not only did spontaneous network discharges last much *longer* than in control cultures, they also consisted of many more action potential which, moreover, exhibited an

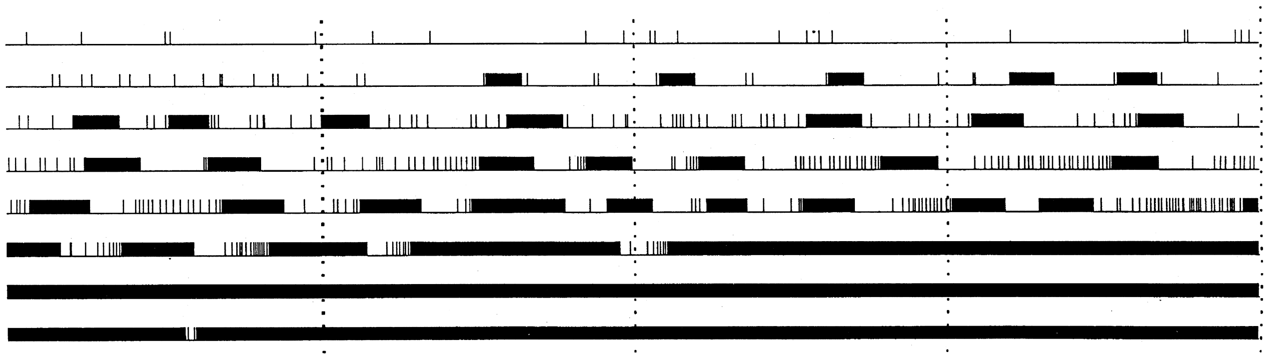

Fig. 14. Continuous recording of action potentials for 1 h (each sweep is 7 min) in a dissociated heart cell culture during the transition from prolonged sporadic contractions to prolonged regular beating.

abnormally strong tendency to be clustered as repetitive high-frequency mini-bursts.

After only 2 weeks in vitro, in contrast, the chronically APV + DNQX treated group showed no more than a modest increase in the incidence of spike mini-bursts. Cultures treated continuously for 2 weeks only with APV, i.e. chronic selective suppression of NMDA-mediated glutamatergic transmission, showed normal levels of SBA even in the presence of APV. After transfer to normal recording medium, they failed to show a noticeable increase above control levels in the incidence of mini-bursts, but network-bursts remained shorter in *duration* and were correspondingly more *intense* than in the other two groups. A 2-week exposure to *DNQX*, i.e. chronic selective suppression of non-NMDA-mediated glutamatergic transmission, led to complete disappearance of SBA (as measured while still in the growth medium) and, following transfer to normal recording medium, to less frequent and less intense burst firing than in control explants.

When either NMDA or non-NMDA synaptic drive was selectively blocked in the course of a recording session (by acute exposure to APV or DNQX, respectively), 2-week-old chronically APV- or APV + DNQX-treated explants showed considerably higher SBA levels than in the controls. In other words, considering the comparable baseline firing levels in all groups when measured in control medium at this age, both of the experimental groups had been rendered relatively insensitive to selective blockade of either of the two main glutamate receptor sub-types. In contrast, acute exposure of 2-week-old cultures to APV after having been exposed continuously

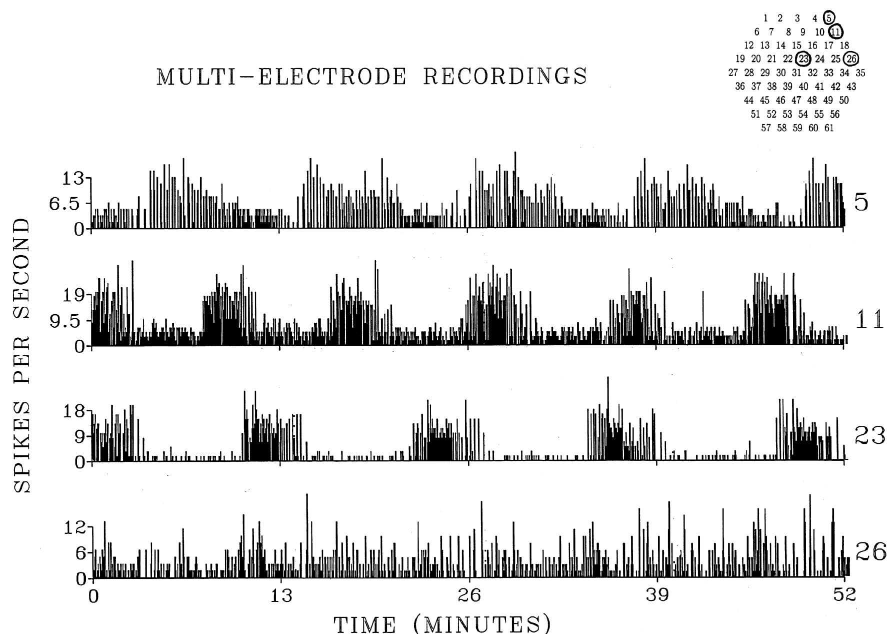

Fig. 15. Simultaneous recording of neuronal firing from four sites on a multi-electrode plate on which rat neocortical cells, dissociated at embryonic day 18, were cultured for ~3 weeks in vitro [504,527].

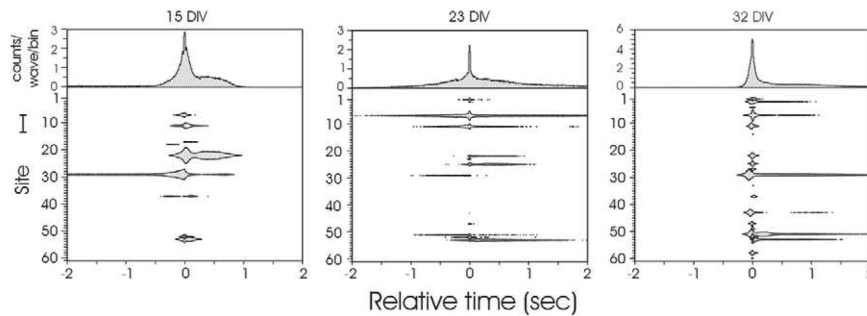

Fig. 16. Spontaneous neuronal firing ('network activity waves') in a dissociated neocortex culture (from an E18 rat fetus) which was cultured for 43 days on a 60-electrode plate, yielding either single- or multiple-unit spike discharges at many of the recording sites at one time or another during the longitudinal registration. Selected 4 h periods at ~2, 3 and 4.5 weeks in vitro are presented, showing the major developmental trends (intermediate days showed a smooth progressive transition, and only minor changes were observed during the last 1.5 weeks that the experiment lasted. The time-bar to the left of each panel indicates a firing rate of 1 spike per wave per 10 ms time-bin at each of the 60 recording sites, while the uppermost traces give (per time-bin) the average number of spikes per wave for all electrodes together. For these calculations, the network activity waves were aligned on the basis of the computed 'center of gravity' for each wave (for full details of the procedure see Ref. [725]).

to DNQX, during development in vitro, consistently eliminated all measurable spike activity.

A striking feature of normal development in organotypic neocortical explants, one which had earlier been noted in *dissociated* neuronal cell cultures, was the presence at many recording sites of slow fluctuations—often quite regular—on the order of a few minutes in the mean firing level. The incidence of such fluctuations was about the same at all ages but a progressive shift was seen, as the explants matured, towards longer cycle lengths and, consequently, to exaggerated minute-to-minute variations in SBA levels. This development was antagonized by chronic exposure (for 2–3 weeks) to APV and/or DNQX, i.e. under conditions of continuous NMDA or non-NMDA receptor blockade, regardless of whether or not a high level of SBA was present. Exposure to APV for only 24 h, while failing to reduce the mean lengths of the cycles, nevertheless made them considerably more regular, whereas DNQX had no noticeable effect. Both at 2 and at 3 weeks in vitro, the slowest fluctuations (i.e. 3–5 min/cycle) were either restored to the 1–2 min range or eliminated altogether by acute inhibition of GABAergic synaptic transmission (PTX treatment). In addition, regularity both in the minute-order fluctuations and in the timing (data not shown) of successive network bursts during high activity portions of the cycle was dramatically enhanced by acute PTX treatment in 2-week-old, but only to a slight extent in 3-week-old cultures.

Our main conclusion is that selective NMDA receptor blockade, despite the persistence of frequent spontaneous spike discharges, produces a comparable 'hyper-activity syndrome'—but one which develops somewhat more slowly—to that resulting from total suppression of bioelectric activity. The crucial role of receptor-linked calcium channels in the development of properly functioning cortical networks will be pin-pointed in the Discussion section, although the possible subsidiary contribution of voltage-linked channels has also been taken into consideration. Spontaneous action potential discharges, by regulating the opening and closing of specific ion channels, thus play an extremely important ontogenetic role already at the earliest stages of neural network formation. This role consists mainly of homeostatically regulating the dynamic balance between excitatory and inhibitory systems, and appears to be accomplished via an impressive variety of activity-dependent mechanisms: the literature implicating (a) dendritic arborization and synapse density, (b) effective inhibitory synaptic drive, (c) number and efficacy of excitatory neurotransmitter receptors, and (d) ion channels controlling trans-membrane current flow is reviewed and discussed. Special attention has been paid to the extreme complexity of quasi-rhythmical patterns embedded, to varying degrees from literally one neuron to the next, within endogenously generated trains of neuronal action potentials, as well as to the very limited insight which we presently have into either their underlying mechanisms or their specific contribution to CNS functional maturation.

Extensive attention is also paid in this paper to the unexpected potential of spontaneous neuronal firing to restore itself to normal levels within a time-frame of hours rather than days or weeks. With NMDA receptors blocked, namely, AMPA/kainate-driven excitatory synaptic drive takes over (albeit with imperfect restoration of the original *patterns* of activity) owing partly to a weakening of GABAergic inhibitory drive and partly to an upregulation of the glutamatergic receptors. It is this (serendipitously discovered) circumstance that makes it legitimate, despite the rapid recovery of high levels of spiking, to interpret the severe long-term effects of NMDA receptor blockade specifically in terms of diminished receptor-, rather than of voltage-linked inward calcium currents. The literature contains some prior examples of such short-term homeostatic processes in neural tissues, and we have reviewed and discussed some plausible underlying mechanisms (see following

sections). Finally, a number of promising directions for further investigation will be examined, and several suggestions made for methodological improvements in cell/tissue-culture approaches to the problem of activity-dependent development of the nervous system.

5. Theoretical evaluation

5.1. Activity-dependent nervous development—methodological aspects

5.1.1. Choice of material

The use of cultured central nervous tissues as model systems for studying physiological development processes is a long-established approach (for an exhaustive review of the early literature, see Ref. [162]) but it will be useful to review here the justifications for continuing this tradition in the present experimental program. One of the most ubiquitous, yet at the same time sensitive, indicators of a healthy functional state in early developing neural networks in vivo is the presence of 'spontaneous' firing of neuronal elements (i.e. bioelectrically monitored activity which originates intrinsically, though as yet incompletely understood mechanisms, within the CNS itself; for partial reviews, see Refs. [52,131,133,208,485,533]). In virtually all regions of the brain and spinal cord which have been examined so far, involving systems ranging from the subcortical control of motor behavior [65,70–72,77,100,128–129,131,138,141, 157,159,171,172,176,201,287–290,298,299,309,337–339, 342,343,374,384,456,472,492,501,509–513,520,521,541–543,570,598,641,662,663,674,702,715,730,741,746] to the generation of slow-waves in the forebrain [5,6,18,19,67, 69,138,139,145,147,151–157,232,245,251,282,308,352,394, 475,545,546,664–669,684,697,737] such activity is characteristically dominated by more or less stereotyped bursts of widely synchronized action potentials and concomitant field potentials, and/or spontaneous calcium transients, usually lasting several tens or hundreds of milliseconds—a primordial '*basic waveform*' coming in a variety of patterns: see Refs. [52,130,131,134,151]; and Figs. 3, 6, 7, 12 and 16—and recurring every few seconds and/or at variably longer intervals [50–54,63,66,87,98,99,109,112,134,140,167,168, 205,209,219,224,240,244,310,322,335,336,340,358,367, 376,385,390,397,408,409,429,432,452,459,465,466,475, 486,515,528,531,597,616,631,664,670,671,690,691,744–746,756–763,773–775,780,781].

This 'burst-pause' mode of firing turns out also to be characteristic of early spontaneous activity in neural tissues cultured in vitro, in every species and from every part of the brain that has been investigated so far [24,25,51,61,88, 89,128,141–143,149,162–165,187,192,194,215,231,235–237,247,253,261,280,281,282,286,318,346,359,365,368,372, 374,392–397,407,430,437,460,480,489,501,504,529,538, 539,551–554,570,606,613,614,673–678,688–695,698,725, 728,745], a circumstance which provides an eminently rational basis for using such preparations as model systems for studying developmental mechanisms in selected neural networks under controlled conditions. Only occasionally are units seen which show an essentially 'tonic' firing pattern, usually irregular but on rare occasions with 'pacemaker-like' regularity with the exception of cerebellar cultures, where this is much more frequent [89,237], and from acute neonatal locus coeruleus slice preparations [112]. Acute supra-optic nucleus slices, too, sometimes show tonic rather than 'phasic' spontaneous discharges [108,109]. The major deviations of in vitro culture models from their intact counterparts seem to concern the neocortex and the spinal cord as they mature, where the cultured tissues seldom display anything approaching the almost continuous sequence of spike-associated field potentials generated intrinsically by large areas of isolated cortex in vivo [50,232,249,251,308,352,667,697] or the prolonged periods of spontaneous firing seen in the acutely isolated intact spinal cord at certain developmental stages [41,113,508–513,541–543,569].

Activity patterns in vitro do closely resemble, however, spontaneous discharges recorded from small pieces of neuronally isolated tissues [56,83,84,100,222,224,225,333, 545,599,744]. Presumably, in a large and densely connected system such as the cerebral cortex, brainstem or spinal cord, activity originating independently at different locations [143,162,192,209,224,271,430,541,542,599,674,678,697, 702], by virtue of its propagation throughout the entire intact network, will re-excite any given recording site more frequently than would be the case with only a portion of it. Small cortical networks are nevertheless quite *capable* of generating continuous discharges which approximate intact patterns, either as individual exceptions ([134,553,721]; also this paper, and see Fig. 7 and Table 8) or as a result of pharmacological enhancement of excitability [221,222, 283,353,490,531,577,599,635]. We nevertheless opted in the present investigation to use the naturally occurring episodic discharges for our (untreated) control groups but, since the developmental consequences of suppressing spontaneous firing are hereby likely to be underestimated, future investigations should consider trying to better simulate the natural levels (as well as patterns) of activity in the intact brain (see below) in their control preparations.

Cultured cortical networks can also show a developmental progression which parallels that described in the lightly anesthetized (thus functionally isolated: [19]) intact neocortex. The initial change, which involves a shift from relatively brief clusters of intense spiking to longer-lasting clusters containing longer interspike intervals [147,156] has recently been observed in organotypic explants in vitro under certain conditions (see Table 8) in addition to the earlier reported dissociated cell cultures ([149,551,553]; also see Fig. 16). Improvements in long-term monitoring of spontaneous bioelectric activity in vitro have also established that a secondary shortening of neocortical burst durations occurs later both in cultured networks [149] (Fig. 16

Table 8
Maturation of spontaneous firing patterns in organotypic rat neocortical co-cultures grown in 'R16' [410] and assayed.in Eagle's 'minimal essential medium' (the *mean firing rate* (MFR) in spikes per second over the entire recording is given as a function of age in vitro; $n = 4$ in all three groups (bold numbers are medians, flanked by quartile values). For each age group, the *period* (i.e. the time elapsing between the onset of successive clusters), and the cluster *length* (in milliseconds), *count* (number of spikes per cluster), and *intensity* (spikes per second during detected bursts) are given for cluster criteria of 1000 ms (defining network bursts) and 100 ms (defining mini-bursts): see Section 2. For the latter, the cluster-period was computed using only interspike intervals <1 s, so as to give an estimation of the internal architecture of a typical network-burst. $*p < 0.01$, Kruskal–Wallis one-way ANOVA)

	1 week	2 weeks	3 weeks
MFR (spikes/s)	0.9–**1.0**–1.1	1.9–**2.3**–2.7	1.2–**1.4**–1.6
CV 60″ (%)	64–**69**–74	65–**111**–156	107–**135**–163
1000 ms			
Period (s)	14–**16**–18	35–**36**–38	14–**15**–17
Length (s)	0.08–**0.44**–0.62	3.9–**5.5**–7.1	1.3–**1.9**–2.6
Count (#spikes)	8–**16**–23	32–**100**–168	10–**18**–25
Intensity (spikes/s)*	36–**61**–86	18–**21**–25	8.1–**9.1**–10.3
100 ms			
Period (ms)	–	528–**555**–587	469–**520**–572
Length (ms)	54–**97**–139	36–**53**–69	57–**62**–66
Count (#spikes)	8–**11**–13	7–**8**–8	4–**5**–5
Intensity (spikes/s)	102–**117**–131	99–**108**–116	71–**76**–81

and Table 8) and in the intact brain [147,156]. These physiological changes are presumably the functional manifestations of concomitant cytomorphological and molecular pharmacological developmental changes (see below), with special attention to the shifting balance between excitatory and inhibitory synaptic mechanisms [15,149,238,424,425, 554,555,726]. Also neuronal membrane properties [768] and glutamatergic synaptic transmission [769] have been reported to follow a developmental time-course similar to that observed in the normal cortex [423–425].

Organotypic mono-cultures, on the other hand, appear to be arrested at a rather primitive stage of physiological maturation (present study). Even when significant changes have been reported with age in vitro [194,261] these have been limited to the first couple of weeks after explantation, and entailed merely the incidence of spontaneously active units and their MFRs gradually attaining levels comparable to what our own (mono-) cultures—not to mention the intact neocortex [147,156,475]—were wont to display from a much earlier point on the developmental time-curve. Evidently, the age at which the tissue is explanted as well as the growth medium and other culture conditions (all of which have differed considerably among the different reports in the literature) are extremely important variables to be controlled in our efforts to achieve the best possible approximation to normal physiological growth conditions, despite the inherently severe constraints of the in vitro experimental design. A major challenge for future work, then, is to be able to quantitatively analyze the relative contributions of diverse structural and molecular factors to the functional maturation of neuronal networks under appropriately permissive experimental conditions.

5.1.2. Treatment effects

Previous studies have shown that chronic suppression of action potential generation, induced by TTX or elevated magnesium concentrations in developing neocortex networks in vitro, leads to a 'hyper-excitability syndrome' consisting of an abnormally high incidence of stereotyped bursts of widely synchronized neuronal discharges [134,149,490,551,555]. The present findings indicate that a similar result is obtained when the suppression of SBA is achieved by means of pharmacological blockade of excitatory synaptic transmission for several weeks [134,711,724] rather than by treatment with TTX or magnesium ions. Chronic high Mg++ treatment had previously been known to induce quasi-epileptiform activity in dissociated *hippocampal* cultures [234,450,612] strongly resembling that reported for neocortex cultures (op. cit.). Since both treatments lead to a considerable reduction in the number of surviving neurons [32,587,588], and since the abnormal firing pattern can be mimicked by acute suppression of GABAergic inhibition [134,149,551], the possibility arises that developing inhibitory neurons are differentially susceptible to the absence of synaptic excitation. Alternatively, as suggested by several studies documenting the dependence of GABA expression upon adequate physiological stimulation (see below), it could also simply be that their phenotypic expression is deficient, resulting in a certain percentage of these cells failing to reach the immuno-cytochemical detection threshold.

An important result in our present study is that 'organotypic' explants, too [25,29,162], are highly susceptible to interference with interference with excitatory synaptic transmission during the early stages of neural network formation, and that this is true despite the lack of detectable cell death [587,588]. This finding becomes especially interesting in view of the fact that *isolated* fragments of neocortex such as were used in the present study have since been shown, in contrast to either dissociated cell cultures [712–714] or organotypic co-cultures in which two explants have been given the opportunity to cross-innervate one another [37], to be arrested in their dendritic maturation despite being electrophysiologically active to a comparable degree ([38]; and present experiments). Presumably, long-range afferent and/or efferent fiber interconnections can exert trans-synaptic trophic effects even in the absence of bioelectric activity [38] and this could be true not only for the cerebral cortex but for other CNS regions as well [771]. That cortical co-cultures—in striking contrast to the isolated explants analyzed in the present study (in which LTP susceptibility [771], NMDA receptors [36] and a variety of proteins [354] also remain at an immature level) but similar to primary cell cultures (see Fig. 16; [149])—show a considerable degree of electrophysiological as well as cytomorphological maturation under control conditions (see

Table 8; [769]) is all the more a reason for preferably using *cross-innervated* fragments of tissue in future organotypic in vitro studies. If the same considerations hold for the spinal cord, it would explain the recent report that, there too, dissociated cell cultures are considerably more excitable and spontaneously active than organotypic (mono)cultures [678].

For practical reasons, quantitative analysis of SBA patterns was carried out on recordings made, not in the medium in which the explants had been cultured, but in Eagle's 'minimal essential medium' (the basal medium from which the growth medium was prepared: [25,29,583]). Qualitative examination of a large set of preliminary experiments had satisfied us, namely, that differences at any age in spontaneous firing in the two media were slight. The fact that, despite enormous variations among the different spike trains recorded in any given group, biologically meaningful (and statistically highly significant) differences between treatment groups emerged even in samples of modest size is an indication that our choice of recording medium did not obscure the major physiological changes induced by the various acute and chronic pharmacological treatments. Some aspects of the variance among spike trains, namely the quantitative characteristics of individual bursts, will be difficult to reduce very much, since it is almost as great among different recording sites within the same preparation (see Fig. 16; [192,271,430,577]) as among the samples taken from different explants, and presumably reflects local differences in synapse number and type as well as in intrinsic membrane properties affecting neuronal excitability. It also needs to be said at this point that a recent quantitative analysis of a small sample of co-cultures (see Table 8) has suggested that, although the same developmental trends were visible in recordings made in the growth medium, firing patterns under these two conditions were not actually identical in all respects (data not shown). It will make sense, then, in future experiments to use the normal growth medium rather than a simplified 'minimal essential' one when physiologically assaying the effects of experimental manipulations on neural development in vitro. Adherence to this caveat will also facilitate comparison of cross-sectional studies involving organotypic cultures with longitudinal studies using primary cell cultures, the latter having now been made feasible by recent improvements in culture procedures (see Figs. 15 and 16; [725]).

5.1.3. Electrophysiological assays

A deliberate decision was made in the present experiments, in contrast to the single unit recordings made earlier in dissociated cultures [149,281,551–555], to use semi-microelectrodes in the interest of greater long-term stability and to accept the fact that most registrations included spikes from two or, occasionally, three units rather than from only one (see Ref. [118] for details of the spike-detection method employed [cf. 181,347]). Although this approach necessarily increases the variance within each sample as far as overall MFRs are concerned, our earlier studies [281,551,750] have indicated that this is a very robust parameter which should not be expected to be particularly sensitive to either age or treatment effects. Indeed, the distribution of MFRs usually proved to be reasonably consistent from one batch of cultures to the next, among different age groups, and between most of the treatment groups. In our earlier work (op. cit.), the tendency for the spontaneous action potentials to cluster in time proved to be the best marker both for normal developmental changes and for deviations induced by experimental manipulations of SBA. Since such clustering is largely a network property (i.e. it has a strong spatial as well as a temporal aspect), some of the burst characteristics registered at different points will necessarily be strongly correlated. This means that, for certain parameters, 'averaging' a few units at a time within a given explant will not appreciably alter the variance over the group as a whole, while greatly augmenting the yield of recordings able to be held long enough to be meaningfully quantified. Indeed, the incidence of spike-associated 'network wave events' and, especially, of mini-bursts within each such event proved to be sensitive indicators of differential maturation under the various experimental conditions.

The criteria for defining what constitutes a spike cluster were somewhat arbitrary in the past (viz. they were little more than 'educated guesses' based on extensive visual perusal of the data) and they sometimes needed to be selectively combined with other parameters so as to define bi-dimensional 'firing categories' before statistically reliable conclusions could be reached. Although such a multi-parameter approach to spike train patterning might need to be implemented in the future, as more and more subtle aspects of neuronal firing behavior become ripe for investigation, the present results have shown that a rigorous application of cluster analysis by itself is capable of revealing developmental and treatment effects even with a limited sample of spike trains from unidentified intra-cortical neuronal sources. More precisely, using a 'sliding' inter-spike interval criterion for defining which spikes constitute a burst and which fall between bursts ([118]; and see Ref. [347] for a critical discussion of this approach), a set of curves, one for each parameter to be examined (see below), could be computer generated for each spike train. Together with the interspike interval histogram, these curves provided an objective basis for selecting optimal criteria for detecting any tendencies for action potentials to occur time-wise in clusters. Such optimization is essential when dealing with such inherently variable data ([294,780]; Fig. 16; differences in burst characteristics among different recording sites within a single cortical network accounts for a large part of the difference from one preparation to the next) if statistically significant results are to be obtained without the need for a prohibitively large sample size. Inter-spike interval criteria for defining what is a burst and which neurons display bursting behavior have often been derived

relative to the MFR of the overall spike train [181,281,347,390,551–554], but this approach renders the definition unsatisfactorily variable from cell to cell as well as precluding the application of multiple criteria in order to reveal spike clustering on different time-scales.

Our newly introduced second-order parameter (viz. the incidence of intra-burst spike clustering, c.q. mini-bursts) proved to be one of the most sensitive ones used up till now for revealing developmental effects of perturbations in ongoing physiological activity. More precisely, it has revealed the unusually high incidence of complex neuronal firing patterns embedded in the spontaneous network bursts of experimentally treated cultures, in contrast to the relatively unstructured and irregular spiking patterns usually seen in untreated control cultures. Since the mere *incidence* of slow-wave associated burst events proved to be a very robust parameter, showing little or no sensitivity to chronic pharmacological treatments of the developing networks, the subtractive method described above was a crucial factor in the statistical analysis of our data. Future studies may benefit from analyzing different classes of mini-bursts (viz. slow, medium and fast) separately but, since no particular frequency class predominated in the present study, the expectation must be that a rather large sample of electrophysiologically or optically [481,482] monitored single-unit recordings, preferably from morphologically identified neurons from specific laminae [11], will be required for adequate quantification of these intra-burst oscillations. Automatic methods for computing the optimal separation points between peaks in the interspike interval histograms [725] should provide a good basis for determining the best values to be used for defining classes of mini-bursts. Such optima have turned out to be by no means identical in all cases, however (data not presented; but see the interval histograms in Fig. 4), so that the relative virtues of trying to accurately fit each spike train separately as opposed to finding an optimal compromise value for characterizing a particular set of cultures, and for comparing different age or treatment groups with one another need to be still worked out.

5.2. Spontaneous bioelectric activity as an independent variable in neural network formation: differential ontogenetic intervention in synaptic interactions

5.2.1. Long-term functional deprivation

Total activity blockade by either TTX or high-Mg++ treatment has been shown in a number of systems to have similar effects. Thus, a comparable reduction in cell survival occurs in cultured neocortical explants under both these conditions [32,588], and dendritic branching can be inhibited in developing cerebellar Purkinje cell cultures using either of these methods for limiting activity-dependent calcium entry [603]. When recorded in normal medium, moreover, chronically Mg++-silenced hippocampal cell cultures [234,450,612] and neocortical or spinal cord explant cultures [134,162] exhibit an 'epileptiform' mode of activity—repetitive short bursts with an abnormally high firing intensity—which resembles that reported in TTX-treated neocortical cell cultures [149,551,554,555]. In a subsequent study [137], a similar paroxysmal discharge pattern was observed in organotypic neocortical explants grown in a high-Mg++-containing medium, thus justifying the conclusion that SBA serves under these conditions to prevent the developing networks from becoming hyper-excitable. Since, on the other hand, similar 'paroxysmal' discharge patterns are characteristic also for normal (i.e. untreated) neocortex cultures at very early stages of network formation [149,162,277,281,380,551,554], the main effect of impaired endogenous activity would in fact appear to retard or prevent functional maturation (see below: *bursting mechanisms*). In intact rats as well, total suppression of neocortical activity by local injection of TTX, or even the reduction caused by prolonged dark rearing, almost completely prevents the normal reduction in duration (and thus potency) of NMDA-evoked excitatory synaptic currents during the first post-natal month in the rat [92]. Nonetheless, firing patterns indicative of even more intense burst activity than in immature controls eventually manifested themselves in chronically TTX-treated cultures [149], so that a secondary effect of the SBA suppression may be to permit the establishment of abnormally strong excitatory drive within developing neural networks [445,490,505,712,713]. Indeed, the intrinsic excitability of visual cortex pyramidal neurons becomes augmented—sodium currents are magnified, and potassium currents diminished—following chronic TTX blockade of action potentials [184].

As was the case with global suppression of synaptic transmission (see above), total glutamatergic receptor blockade led in the present experiments to pronounced hyper-excitability of the cortical explants, thus underscoring the importance of (in this case intrinsically generated) neuronal activity for normal network development, at least under in vitro conditions. Neither after 2 nor 3 weeks, however, did the effects of continuous APV + DNQX treatment closely resemble those seen in our high-Mg++ experiment. Rather than occurring more frequently, network bursts at 2 weeks in APV + DNQX treated cultures tended to be considerably longer-lasting (and to contain more action potentials) than in control explants of the same age. At 3 weeks, in contrast, the bursts were shorter on the whole (though not more intense) than in the control group, so that the higher MFRs measured in the APV + DNQX group can only mean that isolated spikes were occurring abnormally often in the intervals between successive bursts. At the same time, network bursts in these cultures were characterized by an exceptionally high incidence of short spike clusters associated with oscillatory 'mini-potentials', but this particular departure from normality was observed at 2 weeks in high-Mg++ [134,137] as well as in APV + DNQX treated cultures (present study). Since chronic TTX-induced action potential blockade in dissociated neocortex cultures

[149,551,554,555] more closely approximated the abnormalities seen in magnesium-treated organotypic explants [134,137] than in the APV + DNQX treated explants in the present study, the possibility exists that non-glutamatergic sources of intrinsic synaptic activation [317,396,421, 472,486,524–526] could be serving to limit the damage done by suppressing overt SBA.

Of possible relevance to the above-mentioned differences is the fact that in chronic experiments with organotypic cultures [32], the adverse effect of 12.5 μM Mg++ (or TTX) on neuronal survival was considerably greater than with a dosage of 10 μM Mg++ (or APV + DNQX), despite the fact that spontaneous spiking appeared to be completely eliminated in all four cases. Since synaptically mediated membrane fluctuations could be shown to persist at the lower but not the higher Mg++ concentration [32] and since, even in the absence of action potentials, glutamate receptors continue to be activated subliminally by neurotransmitter 'leakage' from synaptic terminals [117,259,457,547,555,636] the implication is that even localized electrical activity, though insufficient to ensure fully normal maturation, can contribute measurably to early cytomorphological [448,547,548] and physiological development of neocortical networks [185,194]. On the other hand, since chronic intensification of spontaneous burst discharges (by means of either GABAergic disinhibition or enhanced excitation) has been shown to lead to opposite physiological effects from those produced by suppressing SBA with TTX [149,175,199,682,704–709, 723], the upper limit for activity-dependent 'plasticity' clearly is not reached by spontaneous firing as it exists in untreated cultures.

In neocortex cultures, such excitation-driven plasticity takes the form of: (i) initial enhancement rather than reduction in the number of dendritic spines [16]; (ii) premature rather than delayed overshoot in the spatial density of pyramidal cell spine synapses [714]; (iii) reduction rather than enhancement of stereotyped burst firing [149,553,723]; (iv) diminution rather than augmentation of evoked synaptic currents [739]; and (v) a decrease rather than an increase in synaptic AMPA currents [704–709]. In spontaneously active cerebellar cell cultures, augmented synaptic inhibition could be demonstrated both electrophysiologically and electron microscopically after several weeks of pharmacologically induced neuronal hyper-activity [614], whereas TTX-induced suppression of spontaneous activity had exactly the opposite effects [613].

The considerable differences resulting from 2, in comparison with 3 weeks of continuous glutamate receptor blockade suggest that the duration as well as the intensity of SBA suppression is an important factor in abnormal functional development. Indeed, a much shorter period of suppression (APV + DNQX for 24 h) not only failed in the present experiments to duplicate the results of chronic treatment, it induced effects of an opposite kind. Thus, although overall firing levels and the incidence of network bursts were hardly affected by overnight exposure to the blocking agents, such bursts were considerably shorter than normal and contained far fewer action potentials, which can only mean that 'background' spiking between successive bursts had become much more frequent. These results indicate, furthermore, that despite anecdotal evidence suggesting that SBA suppression (using TTX) must take place at or near the onset of cortical network formation in order for paroxysmal bioelectric activity to be induced [430], the window of susceptibility to activity-dependent developmental manipulation is in fact quite prolonged. Thus, cholinoceptive neuron survival in spinal cord cultures is reduced in direct proportion to the duration of TTX-induced suppression of SBA for up to 4 weeks in vitro [327], while spontaneous synchronized calcium transients in cultured cortical neurons are enhanced 5 × more strongly above control levels by 3 than by only 2 weeks of TTX treatment [490].

Nevertheless, the fact that abnormal activity persisted in our 3-week-old chronically deprived cultures even after a 24 h recovery period in normal medium should not obscure the possibility that a longer recovery time [9,10,248,332, 404,412,591,592], or else the application of some form of 'therapeutic' stimulation afterwards [93–95,197,204,211–214,303,323,325,448,455,523,568,610,611,633,756,777, 778,781], could have succeeded in reversing the tendency towards paroxysmal firing. It should be noted in this regard, however, that it has so far proven easier, both experimentally [362,712] and theoretically [717,720], to *induce* hyper-excitability at advanced stages of development than to *reverse* it once it has made its appearance. This would suggest that, even though activity-dependent reduction of network-burst intensity [149,553,723] may help to gradually restrict SBA's own 'neuroplastic' efficacy, persistence of stereotyped bursting is not in itself a sufficient condition for the prolongation of 'sensitive' developmental periods.

It is noteworthy that selective blockade of the NMDA subclass of excitatory post-synaptic receptors for 3 weeks resulted in more severe functional abnormalities than did total glutamatergic receptor blockade. Especially striking is the extreme prolongation of the network bursts and their fragmentation into long trains of mini-bursts in chronic APV-treated cultures, in contrast to the pronounced *shortening* of the network bursts (despite equally elevated overall firing rates) in the APV + DNQX group. Evidently, the considerable recovery of SBA in the APV-only group was insufficient to prevent neurophysiological abnormalities from developing, presumably because the voltage-sensitive calcium channels activated during each action potential [59,214,314] were unable by themselves to meet the needs of the developing neuron for this cation [410,446,447]. It should be emphasized, however, that with firing levels which were more closely approximate to those found in the intact cortex [1,19,50,147,200,232, 308,322,475,666–668] the dependency upon NMDA-linked calcium channels for normal development might not be as

great as under standard in vitro conditions. This is indeed suggested by the greater severity of the abnormalities seen after 2 weeks with high-Mg++ [134,137] in comparison with APV-treatment, and could be an argument for ensuring that the control groups in future experiments have a higher SBA level, e.g. by culturing them in a high-calcium/low-magnesium containing medium [430,577]. Conversely, the reduced glutamate concentration adopted for the growth medium in the repeat experiments of Ramakers et al. [555] led to *lower* control SBA levels than in their earlier experiments [551] and, indeed, to less pronounced differences in firing patterns between the experimental and the control groups. Experimentally intensified activity levels, as in the developing frog optic tectum, which generates smaller amplitude evoked potentials following chronic treatment with NMDA [179], might thus, by triggering opposing developmental processes, be able to counteract some of the abnormalities caused by insufficient neuronal excitation during brain maturation [148,476–479].

Since prolonged exposure to DNQX by itself consistently produced opposite effects from those following treatment with APV alone (viz. gradual disappearance rather than return of SBA, leading to depressed instead of enhanced activity upon return to control medium [363]), the two pharmaca might be counteracting each others' effects when used in combination. Examples indeed exist [341] of opposite effects on neuronal function being induced by AMPA vs. NMDA receptor blockade, although changes in the same direction have also been reported [297,448,547,682]. If, in our own preparation, DNQX binding to the glutamate receptor were to have caused sodium and/or calcium channels to become tonically opened [297,405], a situation analogous to high K+ (or veratridine) induced depolarization where despite the elimination of spike discharges [32,91], the effect on the cell membrane is actually to mimic extremely *high* levels of bioelectric activity [21,64,78,91,175,210,214,228, 262,273,407,421,447,523,556,623,626,699,767]—the paradoxical effects of prolonged exposure to DNQX could be accounted for. In line with this possibility is the report that experimentally intensified spike firing in cultured cortical pyramidal cells declines progressively over several days in the presence of bicuculline, eventually falling almost to zero upon return to normal medium [707]. A similar result (but not quite so pronounced) has recently been obtained with long-term organotypic neocortex cultures as well, accompanied by a reduction in dendritic branching of both pyramidal and non-pyramidal cells throughout the cortical thickness [723]. A comparable effect, viz. an 80% reduction in spontaneous AMPA currents, has been reported following 2 weeks of continuous GABAergic disinhibition [411,412], while prolonged *visual* stimulation of the developing cortex in intact rat pups induced a more than fivefold shortening of NMDA synaptic currents [92].

5.2.2. Short-term functional deprivation

The extreme hyper-activity resulting from prolonged selective NMDA receptor blockade cannot be a compensatory response to an overall reduction in ongoing action potential generation, since the initial suppression caused by APV (profound as it was [8,42,54,79–81,134,255,256, 316,326,333,341,356,367,406,419,423,424,490,552,631, 632,703,745,769]) proved to be extremely transient, as short as 2 h at some recording sites [724; and present results] and less than 24 h for the network as a whole. Assay with DNQX established that the excitatory drive was still exclusively glutamatergic, or very nearly so [42,368,490] but was now being carried completely by the *non*-NMDA receptors. The same short-term plasticity phenomenon has recently been confirmed also for cortico-cortical co-cultures (Baker and Corner, unpublished results), a preparation which both morphologically and physiologically more closely approximates normal development in situ ([37,38]; Table 8; [770]) than do isolated explants. A comparably rapid restoration of spontaneous activity despite sustained NMDA receptor blockade has also been described in an embryonic chick spinal cord preparation [42,113]; blockade of nicotinic acetylcholine receptors is similarly overcome in just a few hours [472], but in this particular model system it is the GABA system which in both cases takes over the excitatory synaptic drive.

Conversely, spontaneous calcium currents in cultured amphibian spinal cord cells at the neural tube stage induce large outward potassium currents which, within 24 h, severely reduce the inward current flow [273,313,314, 653,654]. In like fashion, only a few hours of induced burst firing suffices to reduce inward Ca++ currents in lobster stomatogastric ganglion nerve cells and, by so doing, to restore the normal tonic mode of spontaneous firing [706,707]. A similarly brief (1–2 h) period of NMDA-mediated synaptic activity induces, at a critical point in the development of the superior colliculus, a strong decline in the duration of spontaneous synaptic currents [631,632]. In cultured hippocampal neurons, a minute or two of exposure to NMDA is sufficient to trigger a strong reduction within half an hour in the frequency and (to a lesser extent) the amplitude of spontaneous miniature excitatory synaptic currents [47]. Two days of kainate-induced excitation in cultured embryonic chick motoneurons is apparently sufficient to cause, after washout, a sizeable reduction in glutamate-induced currents as compared with control cultures [504].

Strong depolarization (high-K+ treatment) of neonatal rat myenteric neurons for 4–6 h caused a large reduction in the amplitude of transient calcium currents evoked by intra-cellular current injection, an effect which could be reversed in 24 h [228]. A sustained calcium current also became much smaller in the high-K+ medium, but this component required 24 h of depolarization for a maximum effect to be reached nor was it reversible, at least not within 24 h in normal medium. Periodic depolarization of cultured rat neonatal hippocampal neurons, on the other hand, whether by depolarization or by glutamate administration

for 1 h daily, led to a sizeable selective *augmentation* of high-threshold calcium currents [246]. High-frequency electrical stimulation can also induce hyper-excitability, in hippocampal slices from 2- to 3-week-old rats, whereas low frequency tonic stimulation (e.g. 1 Hz continuously for 15 min) induces a prolonged *depression* of physiological responsiveness ('LTD'; [220,230]). The sensitive period for this homeostatic effect is constrained by the maturation of inhibitory synaptic drive, since acute bicuculline treatment renders even older slices susceptible to LTD induction. Finally, bicuculline-induced hyper-activity in dissociated rat visual cortex cultures led within 2 days to a strong reduction in glutamate evoked synaptic AMPA (but not NMDA) currents, whereas suppression of SBA by either sodium channel inactivation (TTX: [707]), membrane depolarization (KCl: [398]) or AMPA receptor blockade (CNQX: [738]) for the same length of time had the opposite effect.

The rapid normalization of SBA levels in the presence of APV (see above) suggested to us the possibility of interpreting the developmental effects of chronic treatment (viz. enhanced paroxysmal discharges) in terms of the selective elimination of NMDA receptor activation per se, rather than merely being a consequence of lowered SBA levels [134,226,295,344,345]. In dissociated visual cortex cultures, too, when treated with various activity blocking agents for 2–3 days, APV was reported to have no lasting effects on either spontaneous firing levels or glutamate evoked synaptic currents [738]. However, quantitative analysis in our explant cultures has revealed that, despite the apparent normalization of overall firing levels, burst parameters measured after 24 h in APV deviate greatly from the control pattern, the bursts being shorter and more intense at all ages than in untreated cultures. Recordings made in APV during *chronic* NMDA receptor blockade, on the other hand, were quite similar to control measurements during the first 2 weeks of treatment, and only after 3 weeks did they come to resemble the 24 h APV pattern of abnormally short bursts. Since this, however, closely resembles the bursting patterns induced by acute PTX treatment, it is highly unlikely (given the *opposite* effects seen following chronic PTX treatment: see above) that it was this abnormal firing pattern which was responsible for the severe functional abnormalities observed in the chronic APV group. Rather, the implication is that NMDA receptor activation is a prerequisite for normal development, at least under those experimental conditions, regardless of the presence—normal or otherwise—of high levels of network excitation.

Spontaneous firing in the present model system thus appears to contribute to cortical network maturation primarily by providing excitatory synaptic input to NMDA receptors [357,403,705], which then homeostatically mediate 'plastic' changes by allowing the passage of calcium ions via receptor-linked as well as voltage-dependent channels [9,10,59,78,117,123,199,262,360,361,410,428,446,447, 490,523,550]. Spontaneous calcium transients appear already at the neural tube stage and are critically involved in morphological (neurite extension), physiological (delayed rectifier K+ current) and pharmacological (GABA phenotype) aspects of neuronal differentiation [62,185,273,313,314,567,653–659]. It is not known if NMDA receptor activation is involved here, but NMDA receptors have been reported to indeed be functional extremely early in neural network development [261,406, 421,490]. In developing neocortex explants, in any event, when NMDA receptors are chronically either blocked (present experiments) or prevented from being activated—either by direct (high-Mg++: [32,137,194]) or by indirect (TTX: [134,551,554,555]) interference with neurotransmitter release—a state of hyper-excitability eventually develops which, upon return to a normal chemical environment, takes the form of excessive spontaneous firing reminiscent of SBA patterns at early stages in network formation (when inhibitory synaptic mechanisms are generally weak in comparison with the functioning excitatory interconnections: [63,217,244,327,380,425,470,515,684]). Although SBA in the presence of APV rapidly attained a persistent 'paroxysmal' character, similar in level as well as in pattern to chronic PTX-induced discharges (see above), the long-term effects of APV on spontaneous firing were just the opposite of PTX. This is thus a further testimony to the developmental impotence of voltage-sensitive calcium channels in the absence of concomitant activation of NMDA receptor-linked channels (but see below).

The possibility can, of course, not be excluded that enough calcium entry can take place under certain conditions to support some degree of activity-dependent maturation even in the absence of NMDA receptor mediation. Indeed, something along these lines has been reported in spinal cord cultures, where stimulus driven plasticity could be restored in the presence of APV by simultaneously elevating the concentration of extracellular calcium ions [213,214,411,412]. Similarly, a sufficiently high concentration of exogenous AMPA can induce receptor internalization even with APV present in the medium, whereas depolarization-induced glutamate release can effectuate this only if the NMDA receptors are allowed to participate [93–95]. Potassium-induced membrane depolarization can actually substitute for NMDA receptor activation in supporting the maturation of fast ('Ia') K+-currents in cerebellar granule cells [262]. Conversely, with TTX blockade of SBA in the same study, NMDA added to the medium was able to substitute for the absence of action potentials. Selective activation of L-type voltage sensitive calcium channels for 1 h is enough to allow met-enkephalin/mRNA expression in cultured spinal cord neurons to continue normally through 2 days of TTX-induced suppression of SBA, but the possible involvement of NMDA receptor activation in mediating some of this protective calcium loading has not been tested [9].

Its beginning, therefore, to look as if many types of developing neuronal networks possess 'homeostatic'

tendencies to attain or maintain a preset level of excitability, not only on the earlier noted ontogenetic scale of weeks [134,160,554,724] but also on a time-scale of days or even hours (for partial review, see Refs. [177,704,709]). Even the partial reduction in adult cortical SBA caused by complete neuronal isolation in vivo (see above) is apparently sufficient to induce a pronounced hyper-excitability of the isolated tissue within a matter of days, which can be counteracted by daily electrical stimulation and is associated with a selective loss of inhibitory neurons [593,594]. Conversely, experimentally induced non-epileptogenic overexcitation of adult neocortex (spreading depression) leads within 1–2 h to a marked up-regulation in the synthesis of trophic factors [369] necessary for the effective functioning of inhibitory interneurons [440–443]. The fact that *endogenous* spike bursts similarly depress rather than enhance cortical excitability—as does tetanic stimulation so as to induce 'long-term potentiation' [e.g., Ref. 125]—is probably simply because, in the absence of external stimulation, excitatory synaptic drive is not normally strong enough to depolarize the neurons past their 'turning-point' [360,361]. A broad scala of mechanisms, furthermore, appear to be synergistically involved (see below) in keeping both spontaneous activity and responsiveness to incoming stimulation within presumably optimal levels for normal species-specific maturation and behavioral adaptation. It is beyond the scope of this review to deal with the burgeoning literature concerning the actual molecular processes triggered within nerve cells by activity-dependent calcium entry, so the remainder of this discussion will restrict itself to those 'downstream' changes which, by directly affecting the response properties of excitable membranes [e.g. Refs. 25,106,221–223,384,418,453,607,635] are most pertinent for satisfactorily explaining the electrophysiological effects described above.

5.3. Spontaneous bioelectric activity as a dependent variable in neural network formation: putative mechanisms underlying spike-train bursting patterns

5.3.1. Short-term neuroplasticity

The rapid return of SBA (within a few hours; also see Refs. [382,383]) despite continued blockade of NMDA receptors cannot have been due to their desensitization, since the restored SBA was completely insensitive to MK-801, but could be completely eliminated by DNQX application, a treatment which produced only a partial reduction of spiking under control conditions [551]. The fact that the initial suppression of ongoing activity by APV in 1-week-old cultures could be counteracted, down to the fine details of burst structure, by concomitant treatment with PTX cf. Refs. [377,424] suggests that GABAergic inhibitory synaptic mechanisms might have become similarly weakened during the overnight period of NMDA receptor blockade, to such an extent that non-NMDA receptors, despite their different kinetics [79,80,81,341,419,423,424,636,769], became capable of sustaining fully normal SBA patterns. When recorded in control medium; furthermore, explants treated for 24 h with APV, either alone or in combination with DNQX (which by itself had no noticeable effect), mimicked at 2 weeks in vitro the abnormally short network bursts displayed by acutely PTX-treated explants of the same age, suggesting that inhibitory drive had indeed been weakened by the overnight NMDA receptor blockade. In 3-week-old cultures, in contrast, PTX-induced disinhibition enormously intensified intra-burst spiking, whereas 24 h APV-treated cultures showed no such effect upon return to control medium. The period of rapid adaptation of GABAergic mechanisms to diminutions in ongoing activity apparently ends for developing rat neocortical tissues sometime during the third week in vitro, which (given the age of the tissue at the time of explantation) would correspond to 3–4 weeks after birth. In all age groups, however, PTX continued to have an enhancing effect on SBA even after overnight suppression of NMDA receptor activation, meaning that a certain degree of GABAergic transmission persisted even when homeostatic adjustments in network excitability took place.

Several examples are to be found in the literature attesting to the relatively short-term dependency of the GABAergic system on adequate levels of stimulation. GABA itself, for instance, induces a down-regulation of the GABA receptor complex within a few hours in acute slices of immature rat neocortex [624,625], while 2 days of TTX suppression of SBA in cell culture leads to a reversible reduction by almost 50% in the number of neurons staining immunocytochemically for GABA [591,592]. Conversely, activation either by glutamatergic synaptic excitation or by veratridin-induced membrane depolarization caused an appreciable up-regulation in the number of GABA receptors [625]. GABA expression in cultured early spinal cord neurons has been convincingly shown, by stimulation as well as by selective deprivation experiments, to respond positively or negatively within a 24 h period according to whether spontaneous inward calcium currents are present or absent, respectively [273,313,314,653,654]. Even in adult monkeys, as little as 4 days of TTX-induced suppression of retinal input leads to a large reduction in the incidence of GABA-positive immuno-stained cells in the visual cortex, which can be reversed by several days of normal visual experience [302,332]. Activity-dependent enhancement of inhibitory responses is possible also in mature rat visual cortex, using tetanic stimulation, although such plasticity is considerably less than in immature animals [230,348,370,371,378]).

It is striking that the mini-burst parameter which was adopted in the present study (i.e. spike clusters based on the 100 ms criterion) unambiguously reveals, both at 2 and at 3 weeks in vitro, an intensity of spike discharges in acutely PTX-treated cortex cultures after 24 h in APV which, even though NMDA-mediated excitatory drive was still suppressed during the electrophysiological assay,

exceeds that in similarly disinhibited control cultures. In the 3-week-old group, moreover, 24 h APV-treated neurons fired in abnormally intense (and correspondingly shortened) network as well as mini-bursts when monitored in normal medium. It is therefore reasonable to consider that, besides any changes in GABAergic inhibition which may have occurred (see above), non-NMDA receptors up-regulated under these conditions so as to partially compensate for the reduction in effective glutamatergic excitation caused by APV. Indeed, 2–3 days of SBA suppression in 1-week-old visual cortex cultures has been reported to lead to a strong enhancement of AMPA as well as of NMDA-mediated spontaneous or evoked synaptic currents, and to a reduction of these currents after a comparable period of experimentally intensified SBA [160,704–709,739]. A mere 1–2 h of spike suppression (with TTX) in developing cortical cell cultures proved sufficient to cause a decline in the rate of removal of AMPA receptors from neuronal membranes, whereas enhancement of SBA (with PTX) led to a change in the opposite direction [199]. APV treatment for 1–2 days in such cultures caused a rise in NMDA (though not AMPA) receptor density [261], an effect which could have contributed to the deviant firing patterns observed in our 24 h-APV experiments (see above). Conversely, quisqualate excitation of acute cortical slices for only 2 h has been reported to induce a down-regulation of AMPA binding at all ages [623], while a few minutes of exposure to NMDA suffices in cultured hippocampal cells to lower the frequency and amplitude of ongoing miniature excitatory post-synaptic currents, concomitant with augmented internalization of AMPA receptors [47,426]. Along the same lines, a single minute of depolarization-induced glutamate release in the same preparation induced an NMDA-dependent increase in AMPA receptor internalization at excitatory synapses [94,95].

Neonatal rat spinal cord cells responded within 72 h to SBA suppression (using TTX, APV and/or DNQX) by (a) enhancement of AMPA-mediated and kainate-induced currents, (b) increased AMPA (but not NMDA) immunofluorescence and accumulation at synaptic receptors, and (c) a reduced turnover rate for AMPA receptors; these effects were fully reversible by 1–2 days in normal medium, whereas opposite effects were observed with artificially *enhanced* SBA [505]. Conversely, NMDA-mediated spontaneous activity in hippocampal slice cultures stimulated the incorporation of a specific type of sub-unit into AMPA receptor molecules which, within 36 h, triggered a persisting decline in excitability caused by the appearance on the scene of inwardly rectifying membrane currents [782]. Cultured hippocampal neurons showed a decline, reaching a plateau already by 15 min following 4 min of 5 Hz electrical stimulation, in the number of AMPA receptors clustered at post-synaptic junctions. This reduction required calcium entry as well as NMDA receptor activation, and was accompanied by a reduction in both the amplitude and the frequency of spontaneous post-synaptic currents [95]. Other types of non-NMDA receptors may also be sensitive to ongoing excitation in a homeostatic way. Thus, even in adult rat neocortical slices, several hours of continuous membrane depolarization was able to induce a down-regulation of muscarinic acetylcholine receptors [626], which are excitatory in this area of the brain [453].

In addition to the likely role for glutamate receptor plasticity in short-term functional homeostasis (see above catalogue of reported effects), further 'downstream' effects on the various ion channels subserving bioelectric phenomena in nerve cells [22,106,119,169,170,273,416,444,449,453, 499,559,566,607,608,652] can also contribute to rapid compensatory increases of network excitability when ongoing neurophysiological activity declines below a critical level. Already at the neural tube stage, within a time window of less than 18 h, cultured spinal cord neurons show a dependence of K + -channel maturation (needed to keep inward current durations within physiological limits) upon normally occurring spontaneous waves of calcium entry [62,185,313,314,653,654,658,659]. Similarly, neonatal rat pyramidal cells in culture respond to 2 days of TTX-induced spike suppression with a large increase of intrinsic excitability as assayed by their sensitivity to intra-cellular current injection [184]. In this case, the effect was shown to be mediated by enhanced sodium (inward) currents in addition to the reduction in potassium (outward) currents, while neither calcium currents nor passive electrical properties of the cell membrane were significantly affected by the suppression of spontaneous spike discharges.

In unspecified neonatal rat brain cell cultures, a variety of sodium channel activators as well as KCl-induced depolarization have been reported to trigger a rapid decline in sodium channel density, and thus in neuronal excitability, which was complete within 2 h and which could be demonstrated experimentally to indeed depend upon inward sodium current flow [175]. High-potassium or NMDA treatment for as little as 2 days induces cultured cerebellar granule neurons to develop larger 'Ia' (i.e. fast) potassium currents than in unstimulated control cells [262] and the former treatment has been reported to rapidly (within a few hours) up-regulate potassium channel expression in neonatal rat superior colliculus neurons [414]. Voltage-sensitive calcium channels, in contrast, can be *down*-regulated by 1–2 days of either electrical stimulation in sensory ganglion cells [400] or by sustained depolarization in neurocytoma cells [183], thus once more working to homeostatically oppose the increased excitation. Similarly, in a completely different model system (the lobster stomatogastric ganglion) experimentally forcing the neurons to fire in repetitive bursts leads within an hour or two to a strong reduction in inward calcium flow during the stimulated spike discharges, probably associated also with an enhancement of outward potassium currents [704–709].

A number of short-term *morphological* effects at the cellular level have also been reported which would be capable of contributing to homeostatic forms of plasticity

in developing neural networks. Neonatal rat hippocampal cell cultures, for instance, showed an age-dependent AMPA- but not NMDA-mediated arrest ('stabilization') or even reversal ('pruning') of dendritic outgrowth which was selective for pyramidal cell dendrites, becoming manifest already within 6 h, and which depended on depolarization-induced calcium currents [410,446,447]. In *Xenopus* spinal cell cultures, too, neurite outgrowth is constrained and guided by calcium influx, which occurs spontaneously in the form of brief transients every few seconds [257], while blocking NMDA (but not AMPA/kainate) receptor activation for 3 days in optic tectum cultures of this same anuran species induces neurite elongation and sprouting in connected, but not isolated neurons [406]. Since blocking not only AMPA/kainate receptors but also sodium action potentials fails to mimic this effect, enough glutamate must be 'leaking' from the axon terminals (see below) to effectively stimulate the NMDA receptors to release whatever 'STOP' signal is the cause of the reduction in presynaptic growth-cone motility. Along the same lines, a 36 h blockade of either NMDA or AMPA receptors, as well as of sodium or calcium ion channels, caused elongation and increased branching of basal, but not apical dendrites in cultured neonatal ferret visual cortex slices [448; also see Ref. 579]. In rodent hippocampus explant cultures, on the other hand, treatment for 3–4 days with either APV or DNQX (and even more so in combination) *reduced* dendritic lengths and also the numerical density of fluorescent labeled excitatory synapses [536]. This difference from other experimental results [116,547,548] might reflect a differential effect of concentration since, in the same type of preparation, a moderate (glutamate-triggered) rise in intra-cellular calcium induced mild dendritic spine *elongation* within 3 h, whereas increased Ca^{++} influx had a much larger effect in the opposite direction [610,611]. In any event, a wide range of rather rapidly acting mechanisms seems to be acting in synergy so as to keep the network's excitability within 'epigenetically' [101,102,134,149,154, 483,653,658,679,753] circumscribed limits.

5.3.2. Long-term structural plasticity

SBA can also affect neuronal morphology over much longer time-scales, as illustrated by the effects of long-term TTX treatment in developing neocortical cell cultures. In this preparation, a quantitative electron-microscopical investigation demonstrated a persistent hyper-innervation of dendritic spine synapses after 5 weeks of continuous SBA suppression, which persisted even after restoration of electrical activity for a full week in normal growth medium [712]. Spine synapse *curvatures* (a putative indicator of the functional state of the synapses [193,747]), in contrast, were predominantly concave, reflecting their state of activation, whether the neurons had been spontaneously active for the full 6-week culture period or only for the last 7 days. Conversely, presynaptic curvatures were predominantly convex, indicating functional disuse, to the same extent in 5-week-old control cultures which had then been exposed to TTX for a week as in cultures in which SBA had been suppressed for the entire 6-week period. Certain structural parameters are apparently equally plastic at all developmental stages, presumably reflecting their instantaneous state of excitation independent of their 'experiential' history [516]. The persistent, selectively excitatory, hyper-innervation of neocortical neurons deprived of SBA during maturation in vitro [134,712,713,718,724] may well have contributed to the physiological hyper-activity characteristic of such cultures [30,149,551,554,565]. This possibility is in line with the suggestion that a transient period of profusely branching short-range recurrent collaterals among developing hippocampal pyramidal cells is causally linked to the pronounced hyper-excitability of that network in the same period: spike bursts are an order of magnitude longer in 10-day-old rat pups than in mature animals, in which the collaterals have become longer and much less branched [258].

It also seems likely that the observed precocious decline in synapses terminating on the dendritic spines of chronically PTX-disinhibited cortical neurons [714,718,724], by tipping the balance between excitation and inhibition in favor of the latter, accounted in part for the reduced incidence of stereotyped burst-pause firing in those cultures [149,553]. Even in organotypic cultures of embryonic neocortex, AMPA receptor activation can rapidly lead to early neurite retraction [540] in accordance with the homeostatic mechanism first proposed by Kater and his group [410,445–447]. It bears mentioning, however, that in organotypic neocortex cultures, prolonged PTX treatment (for 14 days) has been reported to *stimulate* rather than suppress the development of spines on pyramidal cell dendrites [16], while total suppression of SBA in entorhinal–hippocampus co-cultures or hippocampal cell cultures over a corresponding developmental period reduces the number of spines or spine synapses [375,498].

In organotypic hippocampal cultures, on the other hand, spine maintenance is AMPA-dependent but unaffected by chronic TTX treatment, as is also the case with the 'remodeling' of embryonic chick retinal dendrites [761,763], leading to the conclusion that spontaneous vesicular release of glutamate is sufficient to sustain these developmental processes [457]. In another study using hippocampal slice cultures, however, incubation with TTX caused an increase in immature looking spines without a concomitant decrease in the number of mature ones [191,233], thus presumably favoring excitatory drive in yet another example of a homeostatic mechanism operating during neural network formation. Apparently, the 'plasticity-vector' (i.e. enhancement vs. reduction) is not always in the same direction under different experimental and perhaps natural conditions, but this is to be expected on the basis of the bidirectional relationship between intra-cellular [Ca^{++}] and growth postulated by Kater c.s. [410,445–447]. Another possible explanation for apparently contradictory effects is differences

in the exact time-point chosen for the assay [311,712–714,716–718,720,722,724,767].

Chronic SBA blockade in spinal-cord/sensory-ganglion cultures [28,134] results in an 'exuberant' proliferation of regionally non-selective afferent endings (in itself a sign of arrested development: [17,295,406,590,643,645,712,713,717,718]) which in this experiment, similar to the situation reported for *Xenopus* embryo cell cultures that were chronically silenced by growth in calcium-free medium [300] were *less* effective in triggering polysynaptic excitation throughout the cord explant. A very similar degradation of regionally specific innervation has also been reported for cutaneous (though not for proprioceptive [609]) sensory nerve projections to the embryonic chick spinal cord [463]. Yet another example of the appearance of regionally diffuse rather than highly selective axonal branching when SBA is suppressed has recently been reported for layer-6 pyramidal neurons in cultured visual cortex explants. Interestingly, the layer-5 neurons did make selective projections in this experiment even when SBA was blocked, whereas layer-2/3 pyramidal cells failed to make appropriate connections even in bioelectrically active cultures [85,174]. In the intact organism, the precision of optic projection to the superior colliculus, c.q. optic tectum, is similarly degraded either when spontaneous bursts of *retinal* origin are blocked (by intra-ocular TTX injections [558,696]) or when postsynaptic NMDA receptors are locally blocked prior to the onset of functional visual connections [640]. Prenatal suppression of action potentials in intact cat neocortex also leads to a degradation of innervation specificity, this time on the part of the invading thalamocortical axons [97,304]. In somewhat analogous fashion, cerebellar Purkinje cell dendrites in vitro continue to elongate but fail to branch extensively when Ca++-influx is interfered with by suppressing intrinsic bioelectric discharges [603]. The exact same abnormality has been reported for layer-6 pyramidal neurons in ipsilateral rat somatosensory cortex as a result of unilaterally lesioning the locus coeruleus, thereby eliminating the noradrenergic projections [431].

It is striking that glycinergic inhibitory neurons in the gerbil trapezoid body, on the other hand, undergo increased axonal branching following neonatal disinhibition (either by implantation of a strychnine pellet or by contralateral cochlear ablation) and form more extensive terminal boutons [602]. This means that the residual—now imbalanced—excitation coming from the ipsilateral cochlea stimulates rather than constrains the growth of its target cells in this instance but, these being inhibitory rather than excitatory, the effect would once again be to oppose any tendency of the network towards hyper-activity. The enhanced dendritic branching observed on the part of target neurons in the lateral superior olivary nucleus [600,601] would, of course, work in the same direction Similarly, sustained activation of neocortex cell cultures for 2–3 weeks (depolarization by means of 25 mM potassium) induces a visible intensification of immuno-staining in GABAergic neurons, while stimulation by kainic acid enhances the percentage of GABA-positive cells, in addition to their size as measured using confocal microscopy [554]. Conversely, sustained blockade of glycine receptors in embryonic rat spinal cord cultures leads to a reduction in the number of clusters of these (inhibitory) receptors within the soma-dendritic membrane [399].

A number of highly interesting in vivo experiments have further indicated that the morphology of nerve cells is subject to relatively long-term homeostatic regulation by physiological activity which, in all probability, is of intrinsic CNS origin. Thus, TTX implants directly into the thalamus (LGN) of fetal cats induced a threefold increase in synaptic spine density associated with abnormally extensive axonal terminals which, moreover, failed to segregate into layers [173]. Developing optic nerve fibers in *Xenopus* tadpoles become reduced in length by either chronic depolarization (with high-K +) or blockade of voltage-sensitive potassium channels, in both cases apparently because of excessive calcium ion influx [455]. Continuous infusion of TTX directly into the visual cortex of baby ferrets—but not bilateral eye removal—blocks the normal developmental refinement of the initially diffuse long-range horizontal projections which anatomically connect different modules [590]. In an unusual variant on the theme of activity-dependent restrictions upon the development of structural connectivity, localized silencing of the visual cortex (by sustained stimulation of GABAergic inhibitory neurons) in combination with unilateral elimination of retinal input resulted in the active thalamocortical projections being *reduced* in favor of the inactive ones [295]. Nevertheless, morphological up-regulation seems unlikely to account for the gradual development of abnormally intense firing patterns in the tissue culture experiments reported in the present paper, since dendritic branching patterns in such preparations have subsequently been shown to remain frozen in a poorly differentiated state regardless of whether they were active or not [37,38].

5.3.3. Long-term functional plasticity—inhibitory synaptic mechanisms

Deficient inhibitory control is certainly one, if not the most important factor underlying the hyper-activity seen following prolonged SBA suppression in early neural development. Abnormalities in spike firing patterns were apparent in TTX-treated neocortical networks in vitro well before any quantitative morphological differences between the experimental and the control groups could be detected [712,713]. The physiological abnormality in question consisted of a persistent predominance of stereotyped bursting [551] that closely mimicked the SBA patterns typical for very immature networks [281], in which inhibitory synaptic drive is generally weak in comparison with excitatory interactions [162,327,424,425,461]. A similar intensification of spike-burst firing has recently been demonstrated in organotypic occipital cortex cultures as well, following several

weeks of SBA suppression using elevated magnesium ion concentrations in the growth medium [137,194]. Besides the putative deficiency in synaptic inhibition which this implies, the effect might also implicate a persistence of the greater tendency, characteristic of immature neurons, towards intrinsically patterned burst rather than sustained responses when liminally activated [484].

Computer simulation studies have confirmed that addition of synaptic inhibition to a purely excitatory network readily transforms 'phasically' bursting spike trains into 'tonically' firing ones [379] while, consistent with the hypothesis that GABAergic inhibitory development is stimulated by SBA, acute disinhibition of relatively mature neocortex cultures by means of PTX has been shown to cause spontaneous spike activity to revert to a more primitive discharge pattern [149,551]. The same phenomenon has been reported for the chick embryo [670,671]. Furthermore, in contrast to the progressive developmental increase in neuronal glutamate concentrations, GABA concentrations remain frozen at a low level in TTX-treated neurons, and the amount of GABA release which can be provoked by depolarizing stimuli is diminished relative to the release of excitatory amino acids following development in the absence of SBA [554,555]. In neonatal rat cerebellar granule cell cultures, a week of NMDA receptor blockade leads to greatly reduced GABA-a receptor subunit expression, presumably thereby weakening the effectiveness of inhibitory synaptic transmission, whereas activation of the NMDA receptor by its agonist stimulates the development of the GABA receptor subunit [462]. Finally, a strong dependence of GABAergic maturation upon physiological stimulation has also been demonstrated in the *intact* rat, in the form of a selective loss of immuno-positively staining neurons and synapses, along with higher SBA levels and larger receptive fields, in the barrel-field cortex following neonatal whisker ablations [471]. A similar dependence can be inferred for the visual system as well, since orientation selectivity, which depends critically upon GABAergic mechanisms [497,638,755], fails to develop properly under conditions of reduced visual stimulation [103,105, 319,743,749]. Dark-rearing also has the effect of reducing the inhibitory mechanism responsible for paired-pulse depression of evoked synaptic activity [585].

In contrast to the functional deprivation experiments described above, chronic PTX-induced network hyperactivity leads to *less* stereotyped burst firing upon return to normal medium than in untreated cultures [149,553], presumably through a compensatory growth of excessive inhibitory connections [379,614,722]. Both of these suppositions have been directly verified in cerebellar cell cultures, where electrophysiological as well as electron microscopic analysis demonstrated that synaptic inhibition had become reduced in TTX-treated, but enhanced in PTX-treated neurons [613,614]. In analogous fashion, early embryonic (viz. 8–9 days in ovo) chick cerebral neurons show diminished GABA receptor binding and evoked chloride currents after a week of GABA-induced inhibition of SBA [283; also see Refs. 436,458,469,735], while fetal rat spinal cord neurons in vitro respond to either SBA suppression (using TTX) or Ca++-channel blockade by a down-regulation of (inhibitory) glycine receptor complexes in the post-synaptic membrane [362,399]. The normal large developmental increase in the sensitivity of cultured chick embryo spinal neurons to both glycine and GABA is similarly dependent on SBA [461]. Also in the intact rat, 3 months of dark-rearing causes a sizable (~20% overall) reduction in GABA-positive stained cells in all layers of the visual cortex [55], further attesting to the dependence of inhibitory maturation on adequate amounts of stimulation. In a similar vein, abnormal auditory experience in juvenile owls can induce correspondingly abnormal maps of interaural phase relationships in the inferior colliculus, superimposed upon the 'instinctive' one, which is suppressed by activity-dependent GABAergic inhibitory mechanisms [779].

Chronic stimulation of GABAergic receptors (for 6 days) in hippocampal cell cultures leads to reduced interneuronal (GABAergic) cell sizes and NeuroPeptide-Y expression after GABA becomes *inhibitory*, but has the opposite effect at stages when GABA is still acting as an excitatory transmitter [440,443]. In hippocampal slice cultures, on the other hand, it is GABAergic *disinhibition* which leads to over-expression of NPY and somatostatin, while AMPA receptor blockade reduces their expression below control levels [441,442]. Also the expression in visual cortex slice cultures of brain-derived neurotrophic factor (BDNF), essential for the development of inhibitory synaptic mechanisms [441,442], is under the control of calcium influx triggered by SBA acting on voltage-gated, in synergy with NMDA receptor-linked channels [261]. Generalized synaptic blockade in such explants by means of 10 mM [Mg++] for about a month led to a virtual absence of NPY-stained cells, even if several weeks of normal SBA had preceded the treatment [753,754]. As we had pointed out earlier for spine synapse formation [712], the restoration of SBA following several weeks of suppression comes too late to rescue the NPY phenotype. Since the peptide neurotransmitters are expressed specifically in inhibitory interneurons [266,267,736,753,754], these developmental effects of SBA are again of a 'homeostatic' nature which, as we have already seen to be true for GABAergic neurons (see above), appears to be the case in the intact animal as well [507].

In stark contrast with the condition resulting from total elimination of spontaneous spike discharges, chronic selective blockade of the non-NMDA receptors resulted in a gradual disappearance over 1–2 weeks of the residual firing seen both acutely and after 24 h in DNQX. The SBA levels after transfer back to normal medium were correspondingly depressed relative to control cultures. Apparently, non-NMDA mediated glutamatergic receptor activation throughout early neocortical development, far from serving to prevent the network from becoming hyper-excitable (see

above), may be necessary for the maintenance of sufficient excitatory interactions for there to be any spontaneous neuronal firing at all. Such opposing developmental roles of different classes of glutamatergic synaptic receptors would explain the unexpected finding that cultures treated chronically with APV alone showed even higher SBA levels upon return to normal medium than did cultures which had been silenced by a combination of APV and DNQX (see below). It should nevertheless be remembered that non-NMDA glutamate receptor stimulation would, in such a model, only be adding to a process (viz. development of excitatory connectivity) that to a large extent proceeds even in the absence of SBA [26–29,35,135,136,149,160, 162,165,212–214,259,292,293,438,506,551,554,555,613, 712,713].

A serious caveat must be mentioned at this point: DNQX might be binding to AMPA receptors in such a way as not simply to block glutamatergic synaptic transmission but also to cause Ca++ channels to open tonically [297,405], thus mimicking a sustained high level of membrane depolarization despite the suppression of overt bioelectric discharges (see earlier discussion). Prolonged DNQX treatment in this alternative model, then, could in fact be causing a gradual reduction in SBA in part by actually stimulating the maturation of inhibitory synaptic mechanisms (see above), a possibility which was unfortunately not considered in planning the experimental phase of the present investigation. Sustained depolarization, however, is itself a highly ambiguous condition which certainly comes nowhere near reproducing the repetitive transient waves of calcium entry characteristic for the normal physiological situation in early neural ontogeny (see above). Indeed, chronic potassium-induced depolarization of neocortical explants for several weeks in vitro, far from mimicking the developmental effects of TTX or high-Mg++, led to a severe arrest of anatomical and physiological maturation until the tissue was placed back in normal growth medium [31,34]. This intriguing effect suggests that adequate polarization of neuronal membranes as they differentiate may actually be a necessary precondition for neurite extension, receptor insertion and electrical excitability to become possible. The transformation from neuroblast to identifiable neuron at the very onset of neuronal differentiation in amphibians has in fact been demonstrated to depend upon just such a permissive prior development of the chemical machinery for electrically polarizing the cell membrane [467,468].

5.3.4. Long-term functional plasticity—excitatory synaptic mechanisms

Despite the considerable evidence (see above) for an extreme vulnerability of developing inhibitory mechanisms to variations in ongoing levels of interneuronal excitation, careful evaluation of the quantitative aspects of action potential bursts in the present experiments suggests that the abnormalities caused by chronic NMDA receptor blockade cannot be fully accounted for by deficiencies in synaptic inhibition. The most striking deviations from control firing patterns, for instance, were seen at 3-weeks in vitro in the acute PTX- and the chronic APV-treated groups but, whereas network bursts in the latter lasted twice as long as in the controls (while being of comparable intensity, also during the mini-bursts), acutely disinhibited cultures showed strongly intensified spiking throughout burst events of normal duration. In addition, 3-week-old chronically APV-treated cultures showed greatly elevated firing rates over the entire recording session (in normal medium) along with a much stronger tendency than in PTX-treated control cultures for the slow-wave associated spike clusters to be broken up into trains of mini-bursts. Signs of excitatory up-regulation were seen already after 2-weeks of continuous NMDA receptor blockade, inasmuch as mini-bursts were intensified and network bursts prolonged, with neither of these effects being replicated by GABAergic disinhibition. Since virtually identical burst parameters were recorded in these cultures even in the presence of APV, much of the enhanced excitatory drive must have been mediated by *non*-NMDA receptors. Also in our high-Mg++ experiments [137], intensified SBA bursting was revealed by quantitative spike-train analysis which could not be mimicked by GABAergic disinhibition at the same age. Finally, it may be recalled that acute PTX-treatment at 2 weeks in vitro induced network burst firing at remarkably regular intervals, whereas development in the presence of APV (or of DNQX or a combination of the two) had no effect whatsoever on the timing of successive burst events.

The situation after 2 weeks of sustained *total* glutamate receptor blockade is more difficult to interpret: whereas both the chronic APV + DNQX and the acute-PTX groups tended towards relatively discontinuous firing (mini-bursts) throughout each burst event, the bursts themselves showed opposite deviations from control values. Thus, network (but not mini-) bursts lasted considerably longer and tended to contain more spikes in the experimentally deprived group, while in the acutely disinhibited group they were shorter by half in both respects. Even with the NMDA receptors blocked, the chronic APV + DNQX group generated SBA levels and patterns which were virtually indistinguishable from those measured in the controls. The effect of PTX in the presence of APV, on the other hand, was to lengthen and intensify only the short initial (mini-)burst of spikes while drastically curtailing the overall network discharge, suggesting that strong non-NMDA receptor up-regulation rather than diminished inhibition was responsible for the restoration of normal activity. Indeed, SBA in these cultures proved to be largely insensitive to acute blockade of NMDA-mediated synaptic transmission (in contrast to the profound depression of spontaneous activity caused by acute APV treatment in the control group).

APV + DNQX treated cultures were also more resistant than controls to acute exposure to DNQX, although the difference was not as great as with APV, and burst parameters measured with the non-NMDA receptors blocked

were scarcely different from normal. It would appear that these, too, might have up-regulated in compensation for the almost total absence of SBA during early network formation, but one should have expected in that case to see a much greater difference between the APV + DNQX and control groups. Unless, then, there is a rather low ceiling to the maximum level of neuronal activity possible at this stage of maturation (e.g., Refs. [67,134,142,151–153,162,206, 282,380,394,437,661,672,745]) we may suspect that 'downstream' mechanisms affecting all varieties of excitatory synaptic input in similar fashion, such as the various ion channels which regulate trans-membrane currents [13,59, 106,119,167,170–172,175,185,223,262,384,396,416,418, 444,453,499,559,566,607,608,628,639,652,653,667,740, 768] are among the major factors in this type of developmental neuroplasticity.

Also in 3-week-old organotypic cultures in which SBA had been completely suppressed by generalized glutamate receptor blockade (i.e. the chronic APV + DNQX group), the intensity of firing was perfectly normal during bursts of activity but, in striking contrast with the APV-only group, network bursts lasted only about half as long as in the controls. Here too, such bursts nevertheless showed an abnormally strong tendency to be broken up into trains of short-lasting mini-bursts, a phenomenon not seen in acutely disinhibited control cultures. Since the incidence of network bursts was unchanged from the control level, and with each burst containing fewer spikes, the ca. twofold increase in overall firing rates in the APV + DNQX group can only mean that there was a relatively high incidence of isolated action potentials falling in between the computer detected spike clusters. This is reminiscent of the 'supernormal' SBA patterns, i.e. diminished stereotyped burst firing, that was observed in chronically PTX-treated primary cell cultures [149,553]. Precisely the opposite conclusion can be drawn for acutely disinhibited control cultures in the present experiments, viz. a *reduction* of isolated spiking in the intervals between bursts. Also in the chronic APV-only group—similar to what was found for TTX-induced hyper-activity in dissociated neuron cultures [551,554,555]—all or most of the large increase in overall firing rates above control levels can be attributed to a greater number of spikes *within* each network burst. Owing to large interculture variability, and to shifts over time in the fine details of spontaneous firing, none of these points were evident from visual inspection of the time-histograms.

Both mini- and network-bursts in the chronically APV + DNQX treated cultures showed manifest differences in NMDA vs. non-NMDA mediated synaptic drive. Thus, when measured in DNQX, the initial ('mini-')discharge of each network burst lasted longer and contained more spikes than in controls, while the full burst tended to be much shorter in duration but correspondingly more intense. When measured in APV, network bursts in this group were even shorter and more intense than in DNQX, while even the initial spike cluster (mini-burst) was abnormally short and intense. Such differences in the presence of these two receptor blockers would seem to exclude a non-specific mechanism (such as altered ion channel dynamics) as the major factor underlying the abnormal firing patterns which were seen in this group, since any such alteration in general membrane properties might be expected to affect synaptic input in essentially the same way regardless of which excitatory transmitter happens to be operational.

There exist several reports in the literature of just such forms of neuroplasticity as were postulated above on the basis of the quantitative pharmacological 'dissection' of spontaneous spike-train patterns that was carried out in the present series of experiments. The most spectacular of these is a study of the physiological consequences of chronic NMDA + AMPA/kainate receptor blockade for several weeks or months, in cell cultures from five different embryonic rat forebrain regions along with the spinal cord [711]. Upon removal of the blockers from the medium, all of these regions showed virtually unbroken trains of intense synchronized burst firing and waves of calcium entry which can only be described as 'seizure-like', in contrast with the sporadic weaker bursts seen in controls both in normal and Mg^{++}-free medium. This paroxysmal activity was shown, using selective inhibitors, indeed to depend upon sodium action potential traffic as well as trans-membrane calcium flux (but not via the 'L'-type channels) and to be driven by heightened neuronal sensitivity to glutamate. As in our own experiments (this paper), both AMPA and NMDA receptors supported abnormally high firing levels on their own. Voltage-clamp recordings verified the presence of inhibitory as well as excitatory synaptic currents which, when blocked by bicuculline or PTX, caused the spontaneous waves of membrane depolarization to become even more pronounced. It was not established, however, whether such inhibitory activity was equally intense as in untreated control cultures or whether it was in fact weaker (see above), and thus possibly contributed to the observed net increase in SBA.

A more recent report has revealed that in hypothalamic explant cultures an up-regulation of acetylcholine receptors occurs too, so that low-level spontaneous calcium transients appear which can be enhanced by eserine and blocked by atropine or other selective cholinergic blocking agents [51]. This phenomenon could also be demonstrated in cerebellar but not in neocortical cell cultures, so that it seems unlikely that it constitutes a significant factor in our own results. Pointing to the existence of transient populations of cholinergic neurons in certain brain structures, the authors do suggest—conform our interpretation of the earlier functional deprivation experiments (see above: 'inhibitory mechanisms')—that elimination of SBA 'freezes' neural network formation in a primitive condition, and they point out that this constitutes yet another example of homeostatic regulation of developing activity levels (see above). A certain degree of maturity must be reached, however, before this principle begins to apply since, in accordance with the

'biphasic rule' of Kater c.s. [410,445–447], bioelectric activity tends to have a stimulatory rather than a corrective role at the very outset of any given developmental process [31,34,38,75,76,190,311,429,445,468,712,713].

In hippocampal cell cultures from neonatal rats, grown for a week or more in the presence of APV, spontaneous bursts of membrane depolarization are present which (as in our neocortex experiments: this report) rival the intensity of control discharges even when the former are still immersed in the APV-containing medium [253]. In Mg++-free medium, however, the treated cultures show themselves clearly to be more excitable, and also more tightly synchronized, than are the controls. Along the same lines, hippocampal pyramidal (but not GABAergic) neurons showed a fourfold selective increase in NMDA, but not AMPA, sensitive 'hot-spots' along their dendrites after 2–3 weeks of culture in the presence of either APV, TTX or (to a much lesser extent) DNQX [160,404,557]—an effect which slowly reversed itself upon return of SBA. Since the effect of TTX could be neutralized by concomitant exposure to NMDA, sufficient calcium to support neuronal differentiation must be entering even bioelectrically silent nerve cells under suitably permissive conditions, thus underscoring the possibility of preventing or reversing damage done by under-stimulation during sensitive-periods of development.

Conversely, PTX-induced disinhibition was observed to cause a large decline in AMPA (but not NMDA) receptor localization in hippocampal synaptic membranes, an excitability reducing effect which was mediated by spike-driven stimulation of NMDA receptors [411,412]. Hippocampal responses both to locally applied NMDA and to epileptogenic electrical stimulation ('kindling') were enhanced, on the other hand, in adult rats which had been neonatally treated for 2 weeks with MK-801, a selective NMDA receptor blocker [263–265; also see Ref. 454]. An attractive theoretical model has been published [627] in which an intrinsic increase in NMDA receptor *number* interacts with an activity-dependent curtailment of the *efficacy* of those same receptors, mediated largely by the NMDA receptors themselves, to create 'time-windows' for neuro-ontogenetic plasticity. We would only add to this hypothesis that (1) AMPA receptors too, appear from many studies (see above) to be able to adapt themselves in a similarly homeostatic way to ongoing neuronal interactions in developing networks; and (2) AMPA-mediated synaptic excitation, by significantly affecting the patterning as well as the level of background (c.q. spontaneous) bioelectric activity, contributes at least indirectly to the developmental efficacy of the NMDA receptors.

5.3.5. Long-term functional plasticity—intrinsic membrane properties

All in all, then, we have been led by the discussion up to this point to conclude that: (a) not only diminished inhibitory activity but also a variety of synaptic adaptations on the excitatory side are probably involved, in different combinations, in causing cortical networks which suffer functional deprivation during their formative period to become hyperactive in an (only partially successful) 'attempt' to restore (epi)genetically programmed levels of ongoing activity; and (b) NMDA vs. non-NMDA receptor blockade work in certain respects in different directions with respect to the ontogeny of electrophysiological activity, at least for the experimental model system which is central in this monograph. There is, nevertheless, a constant feature in the 'hyper-activity syndromes' reported here as a result of deficient synaptic transmission during early network formation, namely, an exaggerated tendency towards burst-pause firing on a time-scale of fractions of a second, such that relatively long-lasting episodes of spontaneous action potentials become broken up into trains of shorter bursts. Such firing patterns appear not to be due to inhibitory synaptic feedback mechanisms within the network [39,70,71,142,143,159, 162,164,188,255,256,271,282,399,553,673,678,702,714, 723,745] but, rather, probably result in most cases from an enhancement of outward membrane currents (presumably via voltage- and calcium-dependent potassium channels: see below) which become activated during high excitation levels, thus transforming a condition of sustained membrane depolarization into a discontinuous train of action potentials.

We therefore wish to emphasize that, although there are only a few reports in the literature of activity-dependent developmental adaptations by membrane-bound ion channels (see below), their putative contribution to homeostatic or other long-term regulations of functional neural activity certainly deserves to be looked into. Many of the qualitative effects of developmental interference with SBA which were observed in the present study, for instance, seem likely to be due more to changes in inward and/or outward currents in the affected nerve cells than to more global mechanisms such as *receptor* densities, distributions and affinities, *synapse* sizes, numbers and efficacy, and *neuritic* branching patterns, all of which might be expected to mostly affect quantitative parameters (overall firing levels, incidence of network bursting, proportions of activated neurons, etc.). Yet, the present study indicates that it is the patterning of spikes within burst-pause episodes which provides the most striking examples of developmental aberrations, and most of these can probably be best explained by intrinsic cellular mechanisms which transform depolarizing inputs into a variety of self-organized sequences of action potential firing throughout the excited network [13,25,73,99,120–122,187, 209,416,664–668,740]. Any activity-dependent developmental change that affects sodium channel number or kinetics [169,320] seems likely to affect primarily the overall intensity of firing and the duration of network bursts rather than the fine details of intra-burst spike timing. The prolonged bursts recorded in chronically NMDA receptor blocked cultures in the present experiments could therefore implicate up-regulation of the sodium and/or, perhaps more

likely, of the calcium channels which control spike bursting behavior in early development [169,170,607,608, 652,659].

The duration as well as the incidence of propagating spike bursts in developing neurons or networks is constrained by the build-up of 'refractoriness' to further spiking, even of ongoing 'background' activity [114,143,389,677,725,746], which slowly decays over a period ranging from several seconds to more than a minute following the termination of each discharge [59,74,114,128,129,142,143,162,205, 225,238,389,390,490,509–511,514,608,615,661,675,678, 702,725,726,740]. The underlying mechanism has been found in a number of cases to consist of a balance between voltage-gated calcium or sodium (inward) and potassium (outward) currents, respectively, with the burst terminating when the latter comes to predominate [39,123,124,170–172,384,487,607]. Redistribution of intra-cellular chloride could also play a part, however, at least with respect to embryonic chick GABAergic neurons [114]. Chronic TTX-treatment of developing neocortical pyramidal cells in vitro reportedly leads to augmented sodium currents along with diminished sustained potassium currents [184]. Both of these effects would tend to prolong burst discharges, and thus could have been a factor contributing to the abnormally long-lasting network bursts generated by APV-treated explants (with or without concomitant blockade of the non-NMDA receptors) in our own experiments: see above. Given the especially intense bursting which eventually developed when only the NMDA receptors were blocked, despite rapid restoration of high levels of SBA, the implication is that voltage-gated calcium channels by themselves are inadequate under these culture conditions to fully support functional maturation, in particular to ensure an appropriate balance between excitatory and inhibitory mechanisms, and must normally be supplemented by glutamate receptor-linked channel activation.

Repetitive 'mini-bursts', usually lasting several tens of milliseconds, in the course of the more prolonged network burst discharges are a very common (though by no means universal) feature of SBA throughout the CNS from the time of its earliest appearance [40,51,63,70,71,73,109,129–134, 143,170,187,198,201,235–237,253,261,274,277–281,286, 291,317,324,333,335,336,349,355,364,365,390,427,437, 460,508–513,526,529,537–539,541–543,569,570, 581,606,634,649,673–677,690,693,695,697,698,702,765, 772,783]. Here too, an interplay between inward calcium and outward potassium currents probably underlies the oscillation [416,668] but now on a time-scale of several tens or hundreds, rather than thousands of milliseconds. Given the nature of the anomalous bursting patterns induced by prolonged SBA suppression in the present study, in which enhancement of mini-burst activity is the most striking feature, it is precisely these 'medium-length' kinetics which would seem to be most dependent on naturally occurring interneuronal excitation for their proper maturation. For the time being, however, this is purely hypothetical although there is a recent report in the developing cerebellum; it is the fast 'Ia' K + -currents (responsible for determining the duration of the individual action potentials: [384,567,652]) which are enhanced after a week of sustained stimulation, effectuated either through strong depolarization of the cell membrane or through selective activation of NMDA receptors [262]. All in all, it can be recommended that more attention be paid to the ontogeny of, especially, 'medium' duration calcium and potassium kinetics and their important role in sculpting neuronal discharge (see above). It should not be forgotten, however, that the frequency of intrinsic membrane oscillations can be strongly influenced by inhibitory synaptic feedback within the network [87,449,649, 700,701] and are possibly also modulated by rhythmic patterns of spontaneous transmitter release [58,242,321, 340,350,487,488,524,525,581,772,784].

5.4. Appearance and development of multi-second fluctuations in neuronal firing

The frequently observed minute-order fluctuations in spontaneous firing levels deserve some comment in view of their frequent appearance in the developing CNS, in vivo as well as in vitro (for review see Refs. [130,133]). Thus, the earliest behavioral rhythms in amphibians [129–133,172,298,299,343,715], birds [224,288,290,386, 513,522] and mammals [146,178,339,381,494,501,573, 574,575,586,648,685–687,710,733] are driven by intrinsic discharges in spinal cord and brainstem motor networks [114,128,141,143,162,163,342,343,508–512,522,541–543,569,572,576,649,678,685–687,702,727,746] that show a clustering of bursts of synchronized movements into active and (relatively) quiescent episodes which alternate with a periodicity on the order of one to several minutes. Spontaneous motility cycles on a similar time-scale have also been reported in isolated limbs innervated by selected regions of the salamander CNS [741] and in some invertebrates [60,130]. The developing mammalian retina too, shows a comparable periodicity governing the incidence of spontaneous waves of synchronized neuronal activity [217,459,534,757–760,762,763,780,781].

Similar rhythms have also been observed in: EEG amplitude fluctuations in the isolated embryonic chick cerebrum in ovo [67] as well as from the intact human brain [502]; spontaneous spiking in acutely isolated spinal cord [71,492], supra-optic nucleus [108], hippocampus and neocortex [697,745]; induced epileptiform discharges [544] and waves of calcium influx [244,245,526,773] in acute neonatal rat (neo- and/or archi-) cortex slices; and in the mean firing frequency of some lower brainstem and visual cortex neurons in intact rat pups during the first month of post-natal life [140,147]. Multi-second oscillations in firing rate, in the order of 1 min/cycle, have also been noted in various basal ganglia and in lateral geniculate neurons in awake adult rats [12,589]. Normally well synchronized with the rest of the spinal cord, rostrally generated motility slows to ~4–5 min/cycle

in mid-thoracically transected rat fetuses [578]. Since this is a frequency range that, along with a second rhythm closer to 10 min/cycle, has been shown by computer analysis of the spontaneous swimming behavior of anuran tadpoles [132] to be present *simultaneously* with the previously discussed ca. 1-min oscillation, perhaps it is only the relative prominence of multiple co-existing rhythms which is regionally specific within CNS.

This same class of 'minute-order' biorhythms has also been noticed in passing, usually without any attempt at quantification, in a variety of nerve tissue culture model-systems [24,128,143,162,194,271,277,281,286,365,371,393, 480,4693,728,731,772]. Increased prominence of SBA fluctuations in this frequency range has turned out, furthermore, to be one of the most sensitive indicators for changes of cortical activity patterns with age in vitro [281] as well as for arrested maturation in dissociated neocortical cell cultures where SBA had been chronically suppressed [551]. In the present experiments, the *period* of the observed SBA oscillations was observed to increase with age in vitro in an unidentified subclass of cortical neurons, and this developmental change failed to take place either in chronically silenced explants or in bioelectrically active ones in which only the NMDA receptors had been blocked. In addition, as in the above-mentioned cell culture experiments, such treatment strongly reduced the amplitude of the 'minute-order' fluctuations (also following mere overnight exposure to the blocking agents) and, in the 3-week-old group of explants, the resulting enhanced regularity persisted even after allowing functional activity to recover for 24 h in normal medium.

The fact that acute PTX-treatment in our explant experiments caused an even greater increase in the minute-to-minute regularity of spike firing levels than after chronic APV (with or without concomitant DNQX) treatment is consistent with the earlier mentioned likelihood that GABAergic mechanisms are weakened by prolonged NMDA receptor blockade. Some of these fluctuations have indeed been reported to originate from identified inhibitory interneurons [365]. However, since fluctuations on this time scale were still unmistakably present both in the acute-PTX and in the chronic-APV groups, inhibitory synaptic activity clearly cannot be a decisive factor in their genesis. The fact that such rhythms can occur independently in adjacent neuron clusters within a densely connected network (see Fig. 15) suggests that they originate in intrinsic cellular rather than in network properties. The intrinsic 'minute-rhythms' of smooth muscle cells [254] as well as the occasional occurrence of similar oscillations even in isolated cardiac muscle cells (see Fig. 16) and, possibly, also in isolated neurite growth cones in vitro [772] lend support to this supposition. A recent report has documented the existence of locally synchronized spontaneous Ca++ oscillations, in precisely this frequency range, originating from astrocytes within acute rat thalamic slices and capable of intermittently triggering NMDA receptor-mediated depolarization waves in neighboring neurons [530]. A variety of intra-cellular biochemical rhythms have been studied experimentally, and accurately simulated on the computer, which indeed oscillate with a period of one to a few minutes [252,260].

Despite their ubiquity in the developing nervous system, however, minute-order fluctuations on the cellular or systems level appear to have been given little or no systematic attention, even in studies in which a very wide spectrum of 'ultradian' rhythms is extensively discussed [307,417]. It is not even known, for instance, whether they reflect autonomous intra-cellular oscillations [252,260], or whether they result in part from long-tail 'relaxation' effects of the post-excitation refractory mechanisms which were discussed earlier [176,544]. Prolonged depolarizations, usually lasting up to 2–3 min but capable of much longer durations, have also been recorded in brainstem and cortical neurons following bursts of spike generation [221,222,320,376], so that long-lasting enhancement of excitability could contribute in some cases to minute-order activity fluctuations. Higher-order 'circa-horalian' rhythms, such as govern the alternation of sleep states in the intact brain [130,131,337–339,417,456,479,580,598,667–669,730] have been noted in some long-term recordings from neocortical cell cultures along with possible even slower fluctuations in activity levels over many hours or even days [503,725]. The possibility of longitudinal multi-electrode registration throughout development in vitro ought to soon make it possible to chart out the full hierarchy of spontaneous oscillations which characterize the behavior of neural networks, and indeed is predicted by computer-simulated growth models [719,722].

5.5. Activity-dependent nervous development—some pressing questions for further research

As with any model system, conclusions derived from tissue culture studies must ultimately be shown to hold for the intact organism as well (see Introductory Considerations). Otherwise, one may in fact be inventarizing the system's developmental potentialities under extreme experimental conditions, rather than analyzing the factors which contribute to normal ontogeny—an interesting but very different type of project altogether! With respect to bioelectric activity, however, spinal motor as well as midbrain and forebrain visual systems have indeed been shown in vivo to depend on normally occurring SBA for their proper maturation: in both cases, functional deprivation leads to an anatomically demonstrable 'wild growth' of excitatory connections [17,97,116,127,295,304,344,345,463,534,558, 590,619–622,660,680,681,696]. This same conclusion has also been reached for spinal cord [23,26–29,35] and neocortex [134,712,713] tissues in vitro. The results reported here indicate that, in addition to the structural defects, serious *functional* abnormalities can result from inadequate levels of neuronal activity during early development. The

resulting hyper-excitability of an animal's neocortex would predispose it to a variety of behavioral disturbances in later life, presumably ranging from restlessness to 'seizures'. Such effects, which also include hyper-active REM-sleep mechanisms, have in fact been reported following long-term REM-sleep deprivation in neonatal rats [148,306, 474,476–479,734] despite the fact that even a total suppression of this source of SBA would, in these otherwise intact animals, be far from being a complete elimination of endogenous neurophysiological activity [140,147]. Indeed, chronic selective NMDA receptor blockade in neonatal rats is enough to cause a pronounced hypermotility syndrome associated with enhanced cholinergic biochemical activity in the basal ganglia and spinal cord [202,203,732].

Techniques allowing localized manipulation of neuronal activity to be carried out in intact, behaving animals have been more and more employed in recent years, and this is an indispensible long-term complement to in vitro approaches. Thus, intra-cortical injections or slow-release implants of TTX have been studied in infant rats [92,110,111], cats [96,622] and ferrets [590] while muscimol, a GABA agonist, has been successfully injected directly into the cortex of kittens for a week or more at a time [295,296], and so has APV [44,604,605]. APV treatment or, conversely, chronic stimulation of the optic tectum with NMDA has been combined with experimental embryology, viz. the grafting of a third eye in frogs [179,351] in order to study activity-dependent competition effects. A further example of inspired use of a uniquely suitable preparation is the microscopic visualization of neurite outgrowth, and the effects of activity-blocking agents on it, in the intact *Xenopus* optic tectum [115,116,547,548,764]. Prolonged intra-cranial TTX infusion in late fetal cats has demonstrated that, even so early in development, sodium action potentials are crucial for the establishment of appropriate thalamo-cortical terminal arborization patterns [90,96,304]. Intra-ocular TTX injections, in order to suppress intrinsic retinal firing, has also become a favored approach [17,104,105,127,534,558,590,622,680,696] rather than the far less specific enucleation strategy [e.g. 590]. Still more specific pharmacological manipulations, resulting in a selective suppression of ON-center responses, have also been employed using the former technique [103]. Precisely patterned electrical stimulation protocols, in vitro as well as in vivo, have underscored the importance of temporally correlated activity within specified sensory pathways in the competition process leading to precise neuronal interconnections [212–214,680,681,742–744,777]. Combining monocular sensory deprivation with contralateral total suppression of neuronal firing has demonstrated the importance of spontaneous activity per se in morpho-physiologically competitive situations, independent of patterned stimulation [104,560,561].

Conversely, selective manipulation of specific activity generating mechanisms throughout the organism—analogous to the REM-sleep deprivation experiments mentioned above has proven feasible in certain preparations. Thus, chick embryos have been subjected to prolonged continuous NMDA receptor blockade [463] or suppression of cholinergic neurotransmission [278] in ovo. The classical motor paralysis experiments in amphibian larvae (see Refs. [131,269,522]) have been redone, now with proper verification that central as well as peripheral activity has been suppressed, and the earlier conclusions about the high degree of self-organization in neurobehavioral ontogeny could be confirmed and extended [298,299]. An exotically ingenious variant of this approach is the grafting of a larval salamander to a naturally TTX-producing species [292], thus *totally* shutting down the generation of sodium action potentials, in order to study the effects (none were detected) upon innervation specificity. Molecular genetic techniques have recently been used with the same aim, whereby the 'knock-out' of the gene for a single protein, indispensible for synaptic transmission in general, proved unable to produce any noticeable defects (initially at least: massive apoptosis in the second week of gestation wiped out the entire nerve cell population) in neuronal morphology, biochemistry or intrinsic physiological properties [729] NMDA-deficient mutant mice have also been studied using the single gene 'knock-out' approach, again resulting in little or no gross morphological abnormalities [402].

Knocking out the enzyme responsible for GABA synthesis in mice leads to greatly reduced release of that transmitter, and thus to supernormal sensitivity to visual stimulation and loss of neuroplasticity during the 'critical period' of development, but also creates a lifelong potential for renewed plasticity whenever GABAergic inhibition is later experimentally introduced into the organism [204,303]. Knock-out mice have also been used to demonstrate that the activity-dependent enhancement (via GABA receptor stimulation) of inhibitory interneuron size and neuropeptide-Y expression in hippocampal cell cultures is mediated by BDNF [440,441]. Viral infection has been employed to insert specific (AMPA) receptor molecules into the membranes of cultured neurons [782] so it may only be a question of time before this approach, too, finds application in whole-animal studies. Injection of mRNA transcripts (for 'shaker-like' potassium channels) in mouse embryos at the 2-cell stage has in fact already been used for ontogenetic studies [334], while spontaneous mutations in fruit flies have proven useful for revealing that neural excitability is directly correlated with the degree of motoneuron branching in this species [77]. 'Genetic control', however, begins with control *of*, not *by*, the genome (for a discussion of this point see Refs. [134,154,269,276,483,582,679]) and, here too, calcium is deeply implicated, even to the extent that selective gene expression patterns may depend upon via which channels this ion enters the cell [748]. Molecular genetic approaches should therefore, despite their inevitable valuable contributions (e.g. [82,158,391,469,729]), not be seen as a panacea for developmental problems (which, after

all, are fundamentally *epigenetic* in nature [101,102,134, 195,196,213,269,276,293,483,522,653,658,679,752]) but rather, as a welcome supplement to other currently available methods. Similarly, since it will be often desirable for the purpose of analysis to experimentally eliminate certain features of the complete life-support system, e.g. hormonal or long-distance innervation variables—all of the above-mentioned in vivo approaches can probably best be seen as complementary to, rather than substituting for, in vitro (see above) or other types of model systems employing neuronally isolated nervous tissues [150,518,741].

A development of extremely great theoretical importance with respect to activity-dependent neural network formation is the recent attention towards *patterns* of neuronal firing, rather than merely average levels, for optimally stimulating maturational processes [86,149,166]. In cultured *Xenopus* spinal neurons, for instance, the developmental abnormalities caused by low-Ca++ in the growth medium [185,313, 314,653–659] can be effectively reversed by applied calcium pulses only if these are given at or near the naturally occurring frequencies of waves of spontaneous calcium entry into the cells: 20–60 s for GABA expression or K+-channel maturation [739], and ~10 min for neurite retraction [273,392,654,655,658,659]. Spinal ganglion cells respond optimally, by activating specific genes, to brief spike bursts applied at 1 min intervals [211,629], which is a major frequency component of normal SBA (see above), whereas either low frequency stimulation or chronic depolarization were completely ineffective [325]. An earlier report had already indicated that phasic stimulation within the normal physiological range, viz. ~0.5 s spike bursts every 2 s, was optimally effective in halting or reversing neurite outgrowth whereas tonic stimulation had little or no effect [212]. The 'pruning' of redundant neuromuscular synapses in embryonic chicken cell cultures similarly requires phasic (trains of 2 s bursts) rather than tonic stimulation [433]. Still another indication of the crucial importance of *phasic* excitation is the effectiveness of chronic PTX treatment in accelerating both morphological and physiological development in cultured neocortex neurons [149,553,714,723] even though only the patterning (highly regular burst firing) and not the overall level of SBA differed from the controls. Even non-neural cells (lymphocytes) which spontaneously generate calcium waves at ~1 min intervals respond significantly better to this rate of induced calcium entry than to either one-half or twice that frequency [189,401]. The phenomenon has recently been reviewed [78] and its intra-cellular regulation has begun to be investigated [182], as well it should be in light of its great theoretical and, potentially, practical significance for understanding and controlling neurophysiological development.

The ultimate driving force of endogenous neuronal discharges remains to be elucidated. Experiments involving generalized blockade of synaptic transmission have usually failed, except in scattered reports involving neocortical [349], hypothalmic [235], cerebellar [237], lower brainstem [372] or spinal cord [390,678] cultures, to reveal more than incidental putative 'pacemaker' discharges [32,62,165,192, 430,551,554,612]. If neuronal generators such as these exist throughout the CNS, then, they are in most places either very few and far between—and therefore correspondingly potent in triggering propagating waves of excitation—or else consist of small interneurons which have so far escaped detection despite their abundance. In favor of the former possibility is the enormous variation in extracellular SBA levels which is typically encountered from one culture dish to the next (including complete electrical silence) regardless of whether it is explants or primary cell cultures that are being probed ([149,162,281]; and the present experiments). A comparable variance has recently been observed among different recording sites on a 60-electrode plate on which dissociated cortical cells were grown (see Fig. 16; [725,750]), and the use of such multi-electrode plates [192,198,271,272,330,331,430,564,577,595,678,702,725] should greatly facilitate the tracking down of putative 'driver' neurons which continue to fire even after synaptic interactions have been eliminated.

On the other hand, TTX-insensitive excitatory synaptic potentials have been recorded from neurons in such disparate developing systems as *Xenopus* spinal cord [250, 581,636] or optic tectum [547], embryonic chick spinal cord [114], and perinatal rat spinal cord [315,506] or neocortex [80,305,356], while similarly TTX-resistant GABAergic miniature currents have been seen in neonatal rat cerebellar Purkinje cells (triggered by spontaneous presynaptic 'Ca++-sparks' [415,526]) and hippocampal CA3 neurons [317], in guinea pig neo- and paleo-cortex [241], in the fetal rat spinal cord [315] and also in the adult rat hippocampus [524,525]. Furthermore, even extracellular magnesium concentrations which are sufficient to block all spike generation do not necessarily prevent spontaneous membrane potential fluctuations in cultured neocortical neurons [32,43,58,134,241,245,277,341,390, 413,485,490,768,784], all of which indicates that spontaneous release of neurotransmitter is a widespread phenomenon in the vertebrate nervous system, and is probably an important contributory factor in the triggering of spontaneous action potentials [487]. Indeed, spontaneous release of acetylcholine has long been appreciated for the neuromuscular junction [300,321,350,772], and has since been implicated also for glutamatergic synapses in the hippocampus on the basis of spontaneous APV- and Mg++-sensitive membrane currents, which indeed measurably increase neuronal responsiveness to applied depolarizing stimuli [596].

Spontaneous network bursts were consistently absent in cultured rodent brainstem explants, no matter how *electrically* excitable they were, in all cases where extensive probing with a micro-electrode failed to turn up at least one site where spontaneous 'background' action potentials were being generated [142,143]. Even when such sites were abundant, on the other hand, network bursts never occurred

spontaneously in preparations with a high threshold for electrical stimulation, so that localized spiking evidently interacts with network responsiveness [43,349,487,675,746] to trigger widely synchronized repetitive discharges. The failure to appreciate this dichotomy automatically leads to an erroneous overemphasis of the role of 'endogenously active cells' in initiating and patterning SBA discharges [389,390]. Many of the 'background' spikes, of course, may well be themselves driven by stochastic release of excitatory neurotransmitters (see above) rather than being true pacemaker potentials in the sense of, for instance, locus coeruleus neurons [112] and cardiac muscle cells (see Fig. 14). In either case, however, the conclusion must be that the neuronal generators and/or local sensitivities to synaptic activation which make spontaneous propagating bursts possible are inhomogenously distributed across the network, which is also suggested empirically by the observation that different sites can vary greatly in the frequency with which they initiate spreading waves of excitation [209,245,271, 330,430,459,564,678,702]. Consistent, regionally specific, spontaneous neuromotor patterns were obtained from frog neural plate cultures only when large pieces of tissue comprising most of the presumptive area for one of the major CNS divisions were explanted, further attesting to the spatial non-uniformity of physiological differentiation tendencies even *within* a given type of neural network [128]. Also in cultured rat spinal cord [674] and isolated cortical slabs in vivo [697], the frequency and regularity of burst discharges approaches that of the intact animal as a function of the size of the isolated tissue.

Such extreme *inter*- as well as *intra*-network heterogeneity has been shown for small networks on theoretical grounds (with or without activity-dependent interactions in the model) to derive simply from random variations in the way a uniform set of growth rules, which may involve inhibitory as well as excitatory units, becomes realized from one computer simulation to the next [716–721]. A quite different potential source of spatial heterogeneity is the stochastic manner in which pluri-potent cortical precursor cells give rise to highly variable proportions of excitatory and inhibitory neurons [268]. Since small differences in inhibitory drive can have disproportionately large functional consequences [15,385,424,425,563], and since enough synaptic inhibition is present in the neocortex from the onset of SBA, both in vivo [546] and in vitro ([162,164,380,692]; also in the hippocampus: [43,368,427,677]), to be able to influence spontaneous spike-train patterns, even small variations in the excitatory/inhibitory ratio could lead to a large variance among networks which are too small for regional heterogeneities to have been effectively averaged out [128,674,697]. A sparse distribution of putative 'trigger' neurons, electrotonically coupled to spatially variable surrounding domains of spontaneously co-active neurons [121,420,531,532,774,775], has been demonstrated using calcium-imaging in acute neocortex slices taken from rat pups up to 1-week after birth (prior to the onset of cortical SBA: [147,155,322,475]), and the fascinating suggestion made that each such domain goes on to form a structurally and functionally integrated mini-column [774]. Electrotonically coupled, synchronous pacemaker-like firing has also been observed transiently in the locus coeruleus of neonatal rats [112].

Eventual extrapolation of tissue culture results to the fully connected nervous system is constrained by the fact that, by the very virtue of their being neuronally isolated, neocortical networks in vitro have to date been 'models', at best, only for the quiet-sleep state in the intact organism (see above; [232,251,308,352,667,669]). Although there is little or no wakefulness until relatively late in development [130,131,133,146,178,586], much of the time spent sleeping is soon taken up by the 'active' (REM) stage [130,134,145,339,479,580,710], a neurophysiologically more active and qualitatively different state of being altogether [140,337,475,598,730], one which at the cortical level strongly resembles alert wakefulness [337,456,666–669]. The phasic 'burst-pause' patterns of highly synchronized discharges are replaced by a 'tonic' pattern of relatively sustained but irregular and/or high-frequency oscillatory firing which, on the whole, is only locally synchronized [1,200,229,270,491,644,664,665,697]. This change is induced primarily by cholinergic [216,451,453,500,666, 737,766] but also by monoaminergic [4,337,387,418,737] and glutamatergic [668] projections ascending directly from specific brainstem nuclei or from the reticular formation via the thalamus [337,456,667,668]. These transmitters act to reduce one or more of the outward potassium currents and/or to prolong inward sodium currents, thereby transforming repetitive all-or-none bursting into a tonic ('single-spike') mode of firing as well as causing enhanced and prolonged responsiveness, with lowered spike-frequency adaptation, to depolarizing inputs [106,124, 275,284,285,453,538,737]. A recent report suggests that, in addition, non-NMDA receptor-mediated glutamatergic synaptic currents are potentiated while NMDA currents are reduced [218], a combination which would intensify excitatory drive while minimizing the calcium-driven outward membrane currents responsible for 'burst-pause' firing (see above).

Such increased excitability could play an important permissive role in priming the brain to become morphologically and physiologically 'plastic' to sensory stimuli during sensitive periods of development [2,3,46,477,478, 637,638,642,646,647,651]. Such periods themselves represent 'windows of opportunity' for permanent modification of CNS organization, the onset as well as termination of which are controlled by the attained amount of intra-cortical inhibition [204,207,303]. This, in turn, depends upon previous functional activity for its maturation and maintenance (see above), and in early post-natal life the required stimulation is provided mostly by endogenous sources [134,148,474,580,710]. Intrinsic activity of brainstem origin also appears to constrain sensory plasticity in an ex

post facto manner; thus, suppression of REM sleep or of the ponto-geniculo-occipital waves associated with it amplifies the disparity in lateral geniculate cell sizes induced by monocular deprivation in kittens [439,517,617]. The intense spontaneous bursts of neuronal activity during REM apparently serve in this case to homeostatically oppose the growth-stimulating effects of prior visual stimulation, as is also suggested by the significant negative correlation between the amount of REM-sleep in a 6 h period following 6 h of monocular deprivation and the magnitude of the induced ocular dominance shift [227]. Non-REM sleep scores, on the other hand, showed a strong *positive* correlation with the degree of binocular disparity (see Ref. [473]) induced in these same kittens, indeed, the enhancement reportedly equaled the effect of an additional 6 h of monocular deprivation instead of allowing the animals to go right to sleep after the first 6 h [227].

Improved quantitative characterization of emergent 'aroused' firing patterns has also begun to be made [14,669], opening possibilities for realistic in-depth testing of hypothetical mechanisms at the cellular level using mathematical models [379,389,493,650,697,716–722]. Experimental investigation of the possible ontogenetic significance [312,642,646,647] of such aroused vs. the more primitive sleep-like firing patterns (see above) might become feasible by adding amino acid and/or monoamine 'neuromodulators' to the growth medium in an effort to mimic the physiological effects of ascending reticular formation activation [301], possibly facilitated by stimulation via electrodes embedded in the culture dish [198,271,272,331] and using natural patterns of synaptic input [529]. A complementary way of exploiting the in vitro approach would be to co-culture brainstem and/or basal forebrain together with (thalamo)cortical tissues [162,366,537,538] in the hope of obtaining a quasi-natural pattern of neocortical arousal, and then to pharmacologically 'dissect' the contribution of each of the ascending transmitter systems for their contribution to activity-dependent maturation. Since, already at early stages, monoaminergic systems are quiescent during REM sleep but highly active during wakefulness [4,387,388], the relative importance for brain development of these different forms of cortical arousal might also become amenable to experimental analysis.

The theoretical implications of early developmental plasticity as reviewed here are profound, not least because it would mean that neural dependence upon physiological activity begins already at the 'primary process' stage of initial network formation [195,196], and not only once the basic synaptic connections have been laid down. In this initial phase, moreover, it would be the nervous system's own *endogenous* bioelectric activity, rather than stimulation via the sense organs, that provides the driving force for activity-dependent network formation. If the permissive sensory stimulation discovered in the 1960s to be crucial for visual system development in mammals [44,319,619–621,643–645,749] can be regarded as an IMMATURE phase of neuroplasticity, in contradistinction to the MATURE (instructive) forms of plasticity known collectively as *learning and memory* [195,196,348,645,700], the primordial type of plasticity—of intrinsic, c.q. *spontaneous*, rather than sensory origin—which has been emphasized and reviewed in the present monograph could be designated as the PREMATURE phase of activity-dependent neural development. It is only in recent years that this earliest aspect of neuro-ontogeny, after pioneering work by a few groups (see above), has finally begun to attract the widespread attention it clearly warrants [134,208,485,509,522, 655], not least of all because it appears to 'metaplastically' [2,3] set the stage for activity-dependent growth potentialities which are realized only in later phases of development [68,107,230,478,582].

A final word is perhaps in order with respect to the usefulness of model systems and molecular analyses for clarifying questions whose major ultimate interest lies on a higher level of organization, such as integral brain function or animal behavior [134]. Just as biochemical mechanisms directly explain only the morpho-physiological properties of individual cells (properties which themselves have no explanatory value prior to being incorporated into a functioning network), so the significance of neural networks, in turn, can only be appreciated once they are viewed in their proper position within the overall organization of the nervous system. It will have become evident in reading the present monograph that a large discrepancy exists between the extensive demonstration of functional abnormalities at the cellular level, supplemented by putative explanations in terms of molecular mechanisms, and the relatively sparse literature (partially redressed by the present study) on the consequences of such abnormalities for the behavior of the networks in which these defective neurons are components. If the unspoken assumption behind this research imbalance is that, by delving ever deeper into subcellular dimensions, we can get closer to 'ultimate' explanations for phenomena at the level of the entire organism, or even just the nervous system, we would argue [134] that such 'optimism' is totally unjustified. There exists, namely, a hierarchy of explanatory planes in neurobiology—molecular/cellular-network/(sub)system/brain/ behavior—which must be respected, since the mechanisms operating at any given level constitute a causal explanation only for phenomena at the next higher level of abstraction. Even the levels just mentioned will undoubtedly often prove in practice to be too far removed from one another, i.e. too grossly defined, to make possible the successful establishment of causal links between them.

Having said this, it follows that the next step upwards in the explanatory hierarchy, viz. from neural network to central nervous (sub)system, has been even more sadly neglected. How much do we know about what any of the recorded patterns of spontaneous and evoked network activity, whether in the intact brain or in a particular model

system, actually means in terms of *output* signals sent from the structure in question to the target areas which it innervates, and how the various 'downstream' response properties are thereby modulated? In the present context, remedying this omission might mean developing complex co-culture systems outfitted with separate chambers [212,213,496] whereby developing neocortex tissue can be experimentally manipulated while the neural structures with which it forms functional connections in vitro are allowed to develop normally. Abnormal firing patterns reaching these target tissues following the treatment period could then be monitored during artificial 'quasi-waking' states induced in the cortical network by brainstem 'arousal' systems, as well as during the synchronized sleep-like state emphasized in the present paper, and compared with the resulting differences in physiological responses evoked by test stimuli.

Even then, such results will become biologically fully meaningful only when the behavioral and other physiological implications of all of the downstream network activities, both normal and pathological, have also been thoroughly analyzed. Intensified synchronous bursting of neurons in the cerebral cortex, for instance, might be expected not only to induce supersensitivity in some subcortical structures and reduced excitability in others, but also to make it harder for neural systems governing wakefulness to maintain the cortex in the optimally 'desynchronized' state required for alert behavior. Drowsiness and sleep disorders, attention deficits, impulsive motor reactivity, and epilepsy are foremost among the putative clinical problems which immediately come to mind [134,149,180,422,434,435,474, 476,479,551,711,734] but, in light of its possible early ontogenetic etiology, even schizophrenia has been mentioned recently in connection with putative activity-dependent neuropsychological disorder [533]. And, of course, mental retardation in some form or other is the most direct expected neuropathological consequence of all too severe deviations from normal levels and patterns of intrinsic brain activation (for the most recent review of this important topic see Ref. [549]).

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