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Spontaneous neuronal firing patterns in fetal rat cortical networks during development in vitro: a quantitative analysis

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Summary. The development of spontaneous bioelectric activity (SBA) was studied in dissociated occipital cortex cultures prepared from 19 day old rat fetuses. All cultures, recorded one per diem from 5 to 33 days in vitro (div), showed SBA. Computer analysis of 76 extracellularly recorded single unit spike trains was carried out after selection on the basis of stationarity criteria. Statistically significant developmental trends were found in (i) interspike interval dependencies and (ii) fluctuations in mean firing rate, on the order of a minute or longer. The highly dependent firing patterns, including stereotyped bursting, were present mostly in the 9–12 div group, whereas minute-to-minute fluctuations in the intensity of firing were considerably more pronounced in the oldest group (22–33 div) than in the younger cultures. In addition, firing categories defined on the basis of factor-analysis revealed that such fluctuations were almost exclusively to be found in neurons which fired in a pronounced 'burst', rather than a relatively continuous fashion. Only a few mature appearing synaptic structures were observed electron microscopically prior to 12 div, but increased steadily in number thereafter. No cultures prior to 14 div, but all cultures older than this, stained positively for the presence of glutamic acid decarboxylase. An extensive immunoreactive, putative GABAergic, network was present by three weeks in vitro.

Key words: Spike train analysis – Spontaneous activity – Primary culture – Neuronal development – Occipital cortex – Rat

Introduction

The emergence of spontaneous bioelectric activity (SBA) in the course of development of the central nervous system presents an intriguing but complex phenomenon (the term "spontaneous" is used here for action potential discharges which appear not to be triggered by sensory input). Since neuronal excitability (Spitzer 1982) and functional interconnections (Provine 1976) are established very early, putative 'pacemaker' elements for SBA are able to trigger widespread bioelectric activity. As a consequence, conspicuous spontaneous motility is commonly observed in developing animals, starting almost as soon as muscles are capable of contracting. The capacity for SBA to develop is retained in isolated fetal neocortex tissues cultured in vitro throughout the period of normal appearance of functional activities (Calvet 1974; Dichter 1978; Stafstrom et al. 1980) and resembles SBA patterns generated in vivo (see Corner 1985). This makes neuronal culture systems potentially useful for analyzing mechanisms which underly the generation of SBA, as well as for studying its possible role in the normal maturation of the central nervous system.

Previous experiments in our laboratory have shown that, in primary cultures of dissociated fetal rat occipital cortex, an explosive formation of synapses takes place during the second and third weeks in vitro (Romijn et al. 1981). Preliminary observations in the same period showed a development of SBA from more or less 'isolated' action potentials into complex burst patterns. Preparatory

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to addressing the question of the possible importance of SBA for neocortical synaptic development and its functional consequences, it was desirable to obtain insight into the characteristics of SBA at different stages of maturation. For this purpose, we have quantitatively analyzed the in vitro development of spike trains recorded from single units, by means of computation of statistical parameters suitable for describing time series. Since, not unexpectedly, a large diversity of firing patterns was encountered in all age groups, the analysis was extended so as to permit a comparison of the incidence of specified firing categories at different ages in vitro.

Material and methods

Culturing procedure and electrophysiology

Under ether anaesthesia, fetuses were removed from a 19 day pregnant Wistar rat, whereupon the rat was killed by an overdose of Nembutal. After decapitation, the occipital cortices of two fetal brains were extirpated and cut into small pieces. The tissue was dissociated by means of gentle pipetting in nutrient medium, until a suspension of mainly free cells was obtained, and was then inoculated onto a polylysine-coated spot, about 5 mm in diameter, on the bottom of a plastic Petri dish (Nunc, diameter 32 mm) at a density of about 2000 cells per mm^2. Each dish contained about 1.5 ml of medium. Nutrient medium consisted of Eagle's Minimal-Essential-Medium (MEM) with Earle's salts including 2200 mg/ml $NaHCO_3$ (Flow-laboratories), supplemented by 20% horse serum (Boehringer-Mannheim) and without mitotic inhibitors, which was completely refreshed twice a week. The cultures were incubated at 37° C in a 100% humidified atmosphere, automatically controlled to 5% CO_2 in air (which had been verified to keep the pH at 7.4). From 5–33 days in vitro (div) individual cultures were taken (almost) daily, transferred to a vibration-damped recording set-up, and electrophysiologically screened using saline-filled glass micropipettes (tip diameter 1–6 μm) for extracellular recording under phase-contrast microscopy. Cultures were continuously perfused at a rate of 0.7 ml/min with pure MEM, saturated with 5% CO_2 in air, throughout the recording session. The actual recording was started only after perfusion for about one-half hour, which earlier experience had shown to be sufficient for avoiding systematic differences between initial and final recordings. After careful positioning of the electrode onto cell bodies located on top of the glial carpet, or superficially on small reaggregates (mono-/bi-layered), unit activity was stored on magnetic tape (Racal, model Store-7-DS) if, after an adaptation time of 5 min, a signal to noise ratio larger than two had been achieved along with a clearcut amplitude separation from smaller spikes, while the firing rate exceeded 2 spikes per minute.

Morphology

At the end of the recording period, four cultures (one each at 5, 12, 19, and 26 div) were fixed for electron microscopy in 2.5% glutaraldehyde in Na-cacodylate buffer, at 375 mOsm and pH = 7.35, for 1.5 h at 4° C. They were then dehydrated, stained with 1% PTA (Merck) in 100% ethanol at 80° C for 2 h, rinsed with 100% ethanol for 5 min and embedded in Epon 812 (Van Huizen and Romijn 1985). Ultrathin sections (60 nm) were collected on nickel grids with a mesh of 50 × 50 μm. Within several of these holes (each hole was treated as a separate sample) the number of synaptic profiles was counted (Romijn et al. 1981) and, in addition, the percentage of profiles with clearly separated dense projections was determined. On high-power electron micrographs (88,900X) of individual synaptic profiles, the height (Hdp) and the width at the base (Bdp) of the dense projections were measured using a MOP/AM02 semi-automatic measuring device (Kontron, Munich). Hdp/Bdp was then calculated per profile as an indicator for synapse maturation (Van Huizen et al. 1985).

Twelve of the remaining cultures (7–33 div) were fixed in 4% formaldehyde in 0.1 M Na-cacodylate buffer, pH = 7.35 for 2 h at 4° C. These cultures were stained immunocytochemically (ICC, Van Leeuwen 1981) for the presence of glutamic acid decarboxylase (GAD) the enzyme which synthesizes gamma-aminobutyric acid (GABA), believed to be the major inhibitory neurotransmitter in the cerebral cortex (e.g., Dichter 1980). Just before immuno-incubation, the cultures were treated with a mixture of methanol (10%)-hydrogen peroxyde (3%) for about 30 min, and subsequently rinsed in the same buffer as used for ICC, i.e. 0.05 M TRIS in 0.9% NaCl, pH = 7.6. Anti-mouse-GAD (No. 10/30) (Wu 1976), was used (courtesy of Dr. J.-Y. Wu, Baylor College of Medicine, Houston, Texas) in a dilution of 1 : 400 with 0.5% Triton X-100 as a detergent.

Unit sampling

Single unit firing was selected from the taped recordings on the basis of a minimal amplitude jitter and a clear separation from smaller and larger spikes, as estimated from the superposition of consecutive spikes displayed continuously on a storage oscilloscope. Once an unequivocal separation from larger and/or smaller action potentials could be obtained, the unit spikes were converted into standard pulses by means of an amplitude window-discriminator (Mentor, model N-750). These pulses were first stored as time-stamps (DIGITAL: VAX 11/780) and later converted into an interspike interval series. The usable continuous record length for a given unit varied from 224 to 2955 s and comprised 544–5998 intervals. In the program for spike train analysis, non-overlapping stationary samples containing 500 intervals were first selected on the basis of a stationary test ('U-test': De Kwaadsteniet 1982), using a moving window in steps of 10 intervals. This yielded from 1 to 11 samples per unit, with sample-durations ranging from 45 to 1336 s.

Spike train analysis

Spike trains can be represented either as a sequence of times at which successive spikes occur (*'time series'*) or as a sequence of successive interspike time intervals (*'interval series'*), both ways of description being equivalent. Their statistical treatment gives rise to different parameters, however, with each set stressing different aspects of the spike train. Linked with the time and interval series are two stochastic processes – 'Poisson' and 'renewal', respectively – which are characterized by the fact that the corresponding variables (viz., time of occurrence of each event, and intervals between successive events) are statistically independent of one another.

The choice out of available methods for analysis of series of events (Cox and Lewis 1966; De Kwaadsteniet 1982; Nakahama et al. 1977; Perkel et al. 1967) was guided by the following considerations. To begin with, it was evident that the discharge patterns varied from apparently random 'isolated' spikes to highly stereotyped bursts. Slower variations in firing intensity were also noted, fluctuating over periods of several minutes. Based on the

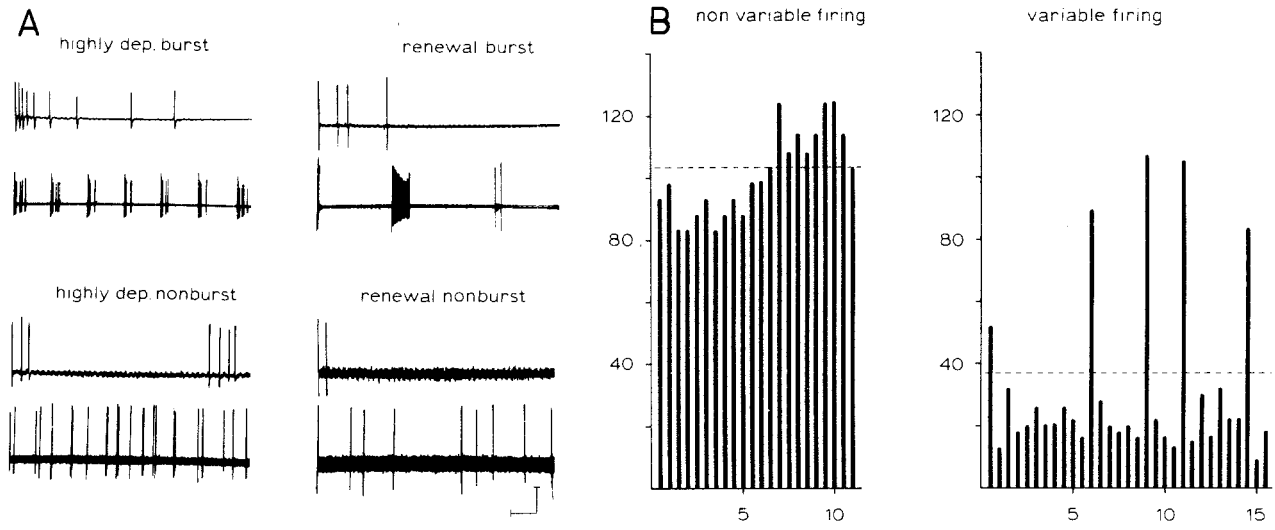


Fig. 1A, B. Representative recordings of spontaneous firing patterns as indicated. **A** Time calibration indicates 100 ms for the upper trace and 2 s for the lower trace of each unit. Amplitude calibration, from upper left and right to lower left and right, respectively: 1000, 200, 100 and 50 μV. **B** Time histograms of a non-variable and a variable firing unit. The number of spike counts per 30 s time bin is depicted on the ordinate (minutes on the abscissa). Broken line indicates the mean per 30 s over the whole recording period

interval series, the spike trains were first of all characterized by parameters which define the overall interval *distribution*. In order to demonstrate deviations from a simple random event-generating process, parameters were also computed which measure *dependencies* present in either the time series or the interval series. The mean values over all samples from a given unit were used in further evaluation of the data.

In separate analyses, using the complete spike train regardless of its stationarity, two additional sets of parameters were computed. First of all, the degree of *burst firing* (i.e., the tendency for spikes to cluster in time) was derived from each interval series (Van Dongen and Bretschneider 1984). Finally, relatively slow *fluctuations in mean firing frequency* were estimated, utilizing the coefficient of variation (CV) of the firing rate per defined time bin, with time bins ranging from 1 s to 120 s.

Statistical treatment

The behavior of the functional parameters during development was first assessed by plotting each of them, in the form of scattergrams, as a function of age in vitro. For statistical treatment of the data, two tests were carried out by means of a computer program: Statistical Package for the Social Sciences (SPSSX-manual, 1983). Unless stated otherwise, two-tailed testing was employed.

1. Correlations between age in vitro and a given parameter were tested in a one-way analysis of variance.

2. In order to deal with redundancy, the degree of dependency among the various parameters was determined using a factor analysis (principal component, with iteration and subsequent Varimax rotation). Main parameters were selected from those sets which were revealed to be independent on the basis of the factor analysis.

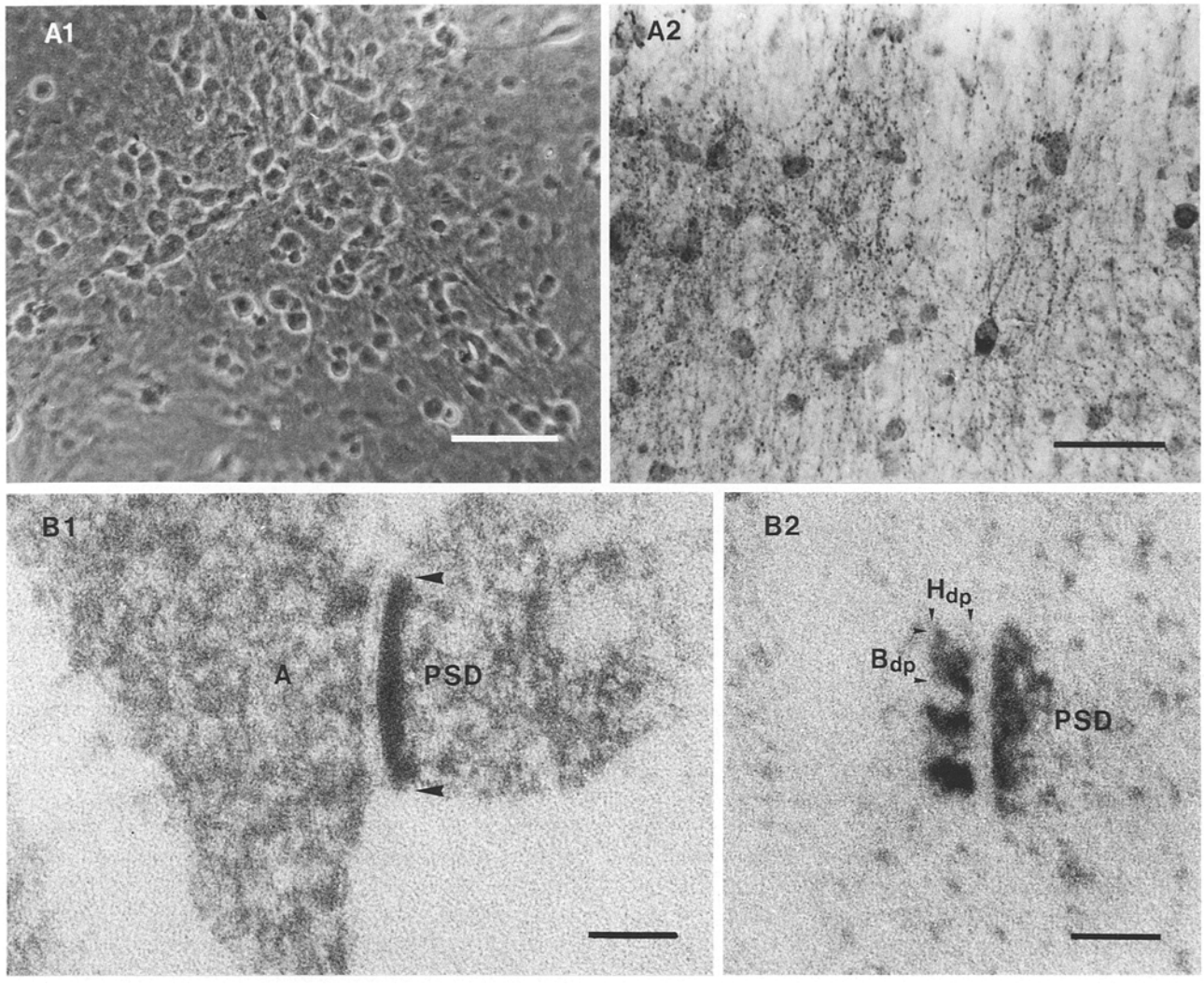
Results

Qualitative morpho-physiological observations

Neurons in twenty-two cultures from a single series were recorded from 5–33 days in vitro (div), and their light-microscopic appearance was noted. At first the isolated cells attached to the Petri dish, and outgrowth of neurites could be observed within a few hours. By day 5, small reaggregates embedded in an extensive network had formed on top of a confluent glial layer. The glial carpet and fiber network thickened during the next week. Reaggregates were only sporadically observed by the end of the second week, however, probably due to glial overgrowth (for further details see Romijn et al. 1981). Abundant neuronal cell bodies (phase-bright; Fig. 2) were observed at all ages, and all of the cultures showed spontaneous bioelectric activity (SBA).

Spontaneous action potentials, in the form of discrete bursts but at rather long intervals (> 20 ms), were first seen towards the end of the first week in vitro (days 5 and 7). The intraburst firing rate ('burst intensity') increased considerable in the next few days. Highly stereotyped burst patterns, i.e., having reproducible sequences of intervals and consistent durations, were present only during the second week – especially on day 9 – while variable spike barrages of longer duration appeared during the third week. In still older cultures, fluctuations longer than 1–2 min were often seen in the mean rate of action potential discharges. Some examples of these firing patterns are shown in Fig. 1.

No cultures up to 14 div, but all cultures thereafter, stained positively for the presence of GAD: at 15, 18, and 20 div GAD-immunopositive cell bodies, as well as some fibers, could be clearly seen. From 21 through 33 div, the cultures displayed an increasingly extensive immuno-positive fiber network (Fig. 2),


A1
A2
B1
A
PSD
B2
Hdp
Bdp
PSD

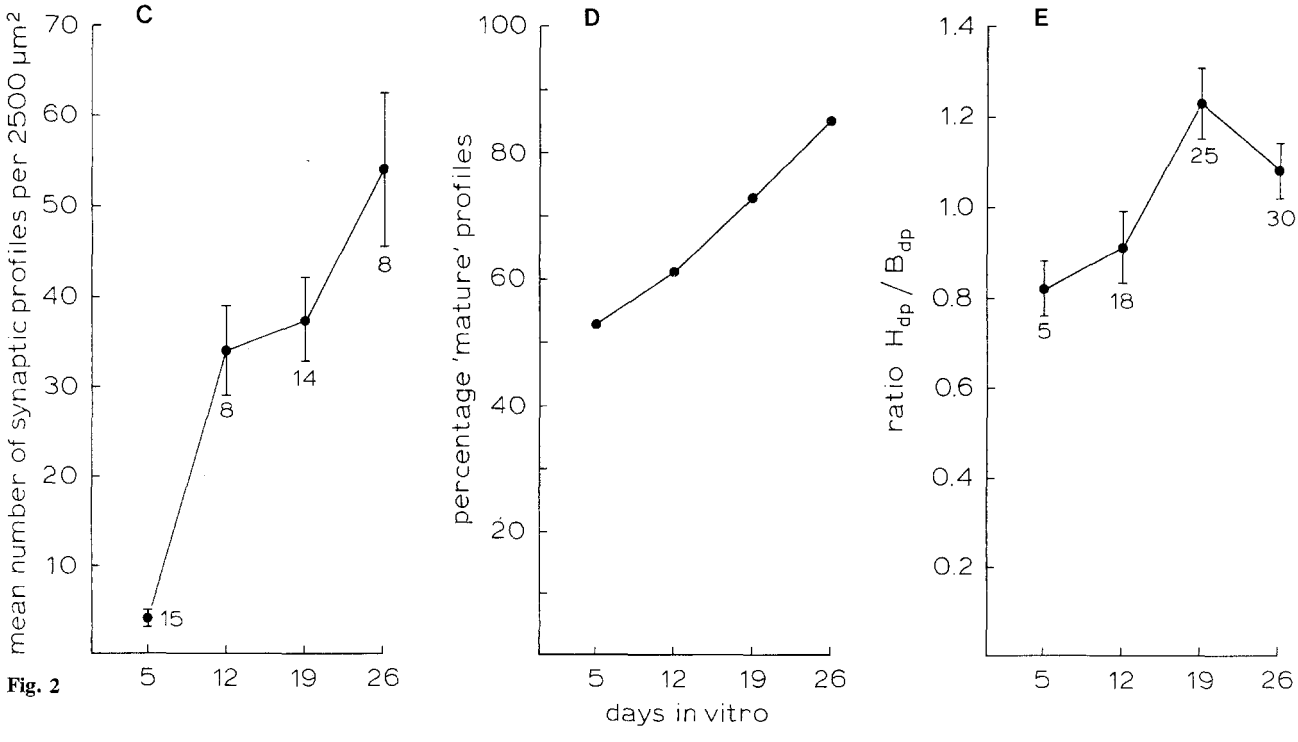

C
mean number of synaptic profiles per 2500 µm²
70
60
50
40
30
20
10
15
8
14
8
5
12
19
26
D
percentage 'mature' profiles
100
80
60
40
20
5
12
19
26
days in vitro
E
ratio Hdp / Bdp
1.4
1.2
1.0
0.8
0.6
0.4
0.2
5
18
25
30
5
12
19
26

Fig. 2

which was mainly observed at the edge of the neuron-rich areas.

Selection of spike train parameters

A total of 24 parameters was computed for each unit (see appendix). Plotting the mean parameter values per neuron as a function of time in vitro revealed, as expected, a large scatter at all ages. The range for each parameter, pooled over all 76 units selected on the basis of the stationarity test, is given in Table 1 along with the age-dependencies. In order to select the optimal parameters for discriminating among spike trains at different ages, a factor analysis based upon all 24 parameters was carried out (SPSSX, 1983). The three first (orthogonal) factors accounted for, respectively, 40, 18 and 11% of the total variance. Several parameters turned out to be highly correlated with each of these factors (see Table 1),thus guiding our selection of the main spike train parameters.

(i) *CV's for minute-order time bins* were strongly correlated with factor 1 (0.94, 0.97 and 0.92 for, respectively, time bins of 30, 60 and 120).

(ii) *Deviation from a Poisson process*, indicating time dependencies (as measured by either the Anderson-Darling (AD) or the Kolmogorov-Smirnov (KS) statistic for departure from a flat spectrum-of-counts) scored, respectively, 0.96 and 0.90 on factor 2. The burst-ratio scored 0.85 on this factor.

(iii) *Deviation from a renewal process*, indicating interval dependencies, was correlated with factor 3. The AD and KS statistic for departure from a flat spectrum of intervals had factor scores of, respectively, 0.96 and 0.92, while the Markov value scored 0.90.

On the basis of these correlations, the following parameters were selected: (i) the *CV per time bin of 60 s*, as a measure for minute-to-minute fluctuations in the mean firing level; (ii) the *burst-ratio* (b.r.), indicating the tendency towards clustering of spikes in time (we chose the b.r. over the time-dependency measures because it provided a direct measure for the degree of spike clustering); and (iii) the *AD statistic for the spectrum-of-intervals*, which registers the departure from independence in successive intervals.

Table 1. Parameter ranges over the mean values per unit (n = 76)

Variable	Factor	Min–Max	age	$P <$
Interval distribution:				
Mean interval (s)		(0.11–2.21)	+	0.05
CV of intervals (%)	2	(40–958)	·	·
Skewness		(0.6–10.8)	·	·
Kurtosis		(1.4–138)	·	·
Gamma		(0.01–6.2)	·	·
AD int. distr.		(0.2–60)	+	0.05
KS int. distr.	1	(0.3–7.0)	+	0.05
Interval/time dependencies:				
AD sp. of cnts	2	(0.8–488)	·	·
KS sp. of cnts	2	(0.7–12.2)	–	0.05
Omega sp. of cnts		(0.7–14.6)	·	·
AD sp. of int.	»3«	(0.2–166)	–	0.005
KS sp. of int.	3	(0.3–7.8)	–	0.001
Markov value	3	(0.003–0.76)	–	0.0001
Markov order		(0–27)	–	0.01
Bursting parameters:				
Burst duration (s)		(0.08–1.67)	·	·
Burst period (s)		(0.6–44)	+	0.05
CV burst period (%)		(5–141)	+	0.0001
Burst ratio	»2«	(1–81)	·	·
Burstint. (spikes per s)		(1.3–59)	·	·
Minute-order fluctuations:				
CVTMBN 1 s (%)		(15–601)	+	0.05
CVTMBN 5 s (%)		(6–441)	+	0.005
CVTMBN 30 s (%)	1	(5–190)	+	0.0005
CVTMBN 60 s (%)	»1«	(3–185)	+	0.001
CVTMBN 120 s (%)*	1	(3–136)	+	0.0005

Correlation with age, positive (+) or negative (–), is indicated whenever a statistically significant age effect could be concluded from a one-way analysis of variance, on the basis of 7–12, 13–21 and 22–33 div age groups. A variable is listed as scoring on a given factor (see text) if the correlation coefficient is greater than 0.80.
»·«: Parameter used as discriminating variable
*: (n = 74)

Definition of functional categories

Using an approach borrowed from Shtark et al. (1976), the 76 units were sorted into 12 mutually

Fig. 2A–E. Light-microscopic appearance and quantitative electron microscopic observations. **A1** Phase-contrast picture of a fixed culture at 13 div. **A2** Bright-field picture of GAD-stained culture at 22 div (bar = 50 μm). **B1** Electron micrograph of a synaptic profile without any presynaptic dense material at 12 d (bar = 0.1 μm. **B2** Synaptic profile with fully developed dense projections at 26 div). A = axon, PSD = postsynaptic density (for Hdp and Bdp, see Methods). **C** Numerical synapse development; data represent the mean, standard error and the number of samples. **D** Percentage of synaptic profiles with clearly separated presynaptic dense projections; data represent percentage of the total number of synaptic profiles per age. **E** Maturation of presynaptic dense projections; their appearance is expressed as the ratio of the height to the width at the base (see Methods). These numbers apply only to profiles having clearly separated presynaptic dense projections; data represent the mean, standard error and total number of 'mature' synaptic profiles

Table 2. Distribution of units over firing categories at different ages (see Results section for definitions of the criteria used). The proportions are given of the total number of units (n =) in each age group. Abbreviations: REN – renewal process; MOD – moderate; HI – high interval dependencies

	Continuous						Variable					
	Non-burst			Burst			Non-burst			Burst		
Dependent	REN-	MOD-	HI-	REN-	MOD-	HI-	REN-	MOD-	HI-	REN-	MOD-	n =
7–12 DIV	–	0.13	0.30	0.13	0.26	0.17	–	–	–	–	–	23
13–21 DIV	0.13	0.36	0.13	0.06	0.13	–	–	0.10	0.03	0.07	–	31
22–33 DIV	0.27	0.18	–	–	0.05	–	0.09	0.05	–	0.23	0.14	22

exclusive firing categories, on the basis of the defining values indicated below. Selected recordings of single spike trains, illustrating four visibly different firing patterns and their classification, are shown in Fig. 1.

Non-variable firing is defined if the mean firing rate per 60 s epochs had a coefficient of variation equal to or smaller than 30%.

With b.r. < 5, we then obtain the following three ('non-burst') categories:

(1) *Renewal firing*, i.e., quasi-random firing pattern characterized by a lack of serial dependency. Units were put into this category if the AD statistic of the spectrum of intervals was less than 3.86, the level at which the renewal hypothesis is refuted at the 99% confidence level.

(2) *Moderately dependent firing*, i.e., a mode of firing characterized by an AD statistic (for spectrum-of-intervals) equal to or greater than 3.86, and less than 15.

(3) *Highly dependent firing*, i.e., firing characterized by a large serial dependency, i.e., the AD statistic for the spectrum-of-intervals is equal to or greater than 15.

Categories 4–6 (viz, non-variable, *burst firing)* are defined as above, but with b.r. ≥ 5.

Categories 7–12 (viz., *Variable firing*) follows the same definitions as 1–6 except that the mean firing rate per 60 s epochs is associated with a CV larger than 30%. Note: in the present series there were no variable firing units which showed highly dependent burst firing, so that this category is not listed in the heading of Table 2.

Neuronal firing patterns at different ages

The definition of three, relatively homogeneous, age groups was based on the age-dependent behavior of the CV for 60 s time bins and of the AD statistic for the spectrum-of-intervals, as reflected in their respective scattergrams (not shown). The distribution of firing categories over these three groups (Table 2) clearly indicates the developmental trends, viz., decreasing interval dependencies and increasing minute-order fluctuations, already noted in Table 1. Bursting expresses itself in the form of developmental changes only in conjunction with the other two parameters (Table 2). Finally, it should be noted that the highly significant increase with age in the variability of burst occurrences (Table 1: CV burst-period) is a reflection of their relatively consistent timing at 9–12 div.

Units in the 7–12 div group were characterized by (i) a high incidence of strongly interval-dependent firing patterns, (ii) 'burst' firing in about half of the units and (iii) a total absence of 'variable' firing patterns. In the 13–21 div group, in contrast, (i) most of the units show only moderate interval-dependency, (ii) fewer units reach our burst criterion, and (iii) several units display highly variable firing patterns. In the 22–33 div group, finally, (i) a further decrease in interval dependency is noted, with most units in fact showing renewal patterns, and (ii) fully half of the units now display strongly fluctuating (i.e., 'variable') firing from one minute to the next. It is striking that 8 out of 9 'bursters', but only 3 out of 13 non-bursting neurons, show these large minute-order fluctuations in firing rate between 22 and 33 div (Table 2).

Quantitative electron-microscopic observations

The EPTA staining technique clearly showed synaptic densities, especially presynaptic dense projections, against an almost unstained background. Although the contrast in these preparations was not quite as good as can be achieved with in vivo material, quantification could still be done reliably (Van Huizen and Romijn 1985). There was a large increase in synapse density between 5 and 12 div, followed by a second increase after 19 div (Fig. 2). The ultrastructure of the synapses also matured

during this period but with a different time-course: the percentage of profiles with clearly separated dense projections increased progressively from 5 to 26 div (Fig. 2), whereas the ratio Hdp/Bdp increased mainly between days 12 and 19 (Fig. 2).

Discussion

Despite large interneuronal variability, single unit activity patterns in an age-series of cerebral cortex cultures were able to be statistically evaluated using analyses-of-variance, backed up by factor-analysis. The most useful parameters for demonstrating developmental changes proved to be those which (i) revealed the existence of sequential patterning within the trains or (ii) estimated the relatively long-term fluctuations in firing rate (ca. 1 min or longer). Three distinct periods of functional development in vitro could be defined: (a) an *early* phase (up to ca. 12 div) characterized by regular, often highly stereotyped, bursts and strong dependencies between successive intervals, (b) a *middle* phase (ca. 13–21 div) during which clearcut bursting has largely disappeared and, on the whole, only moderate interval dependencies are encountered, and (c) a *late* phase (ca. 22–33 div) in which renewal (i.e., interval-independent) firing patterns predominate, often in the presence of large fluctuations in mean firing rate, over periods of ca. 1 min or longer. Subsequent analysis of these age groups in terms of 12 firing categories, derived from the three parameters which contributed most to the overall variance, enabled us to confirm these impressions. This analysis indicated, further, that minute-to-minute fluctuations in neuronal activity were strongly correlated with the tendency for action potentials to be clustered in time. It is interesting to note that such 'bursts', in themselves, showed no significant developmental changes in burst *duration* (varying from 80 ms to 2 s), *size* (varying from 1 to 80 spikes ber burst) or *intensity* (varying from 1 to 60 spikes per s). The only developmental change noted in the bursts was a slight decline in their *incidence* (overall range, ca. 0.5 to 45 s between successive bursts). Since afferent impulse traffic into the neuronal network under the relatively constant conditions existing in vitro appears to be negligible (Corner and Crain 1972), all of the above-mentioned developments presumably reflect alterations in intrinsic neuronal response properties and interneuronal connectivity.

In the present study a very large increase was found in synapse numbers between days 5 and 12, which corresponds closely to the time during which SBA appears and rapidly evolves from sporadic spikes and low intensity, irregular, bursting to stereotyped bursts with relatively high firing frequencies. In view of the fact that the cultures showed no substantial increase from day 5 to day 12, either in the percentage of profiles with distinct dense projections or the ratio Hdp/Bdp (these being ultrastructural indicators for synaptic maturity: see Dyson and Jones 1976), the numerical increase per se would seem to play an important role in the early electrophysiological development of the network. Since the 9–12 div period is characterized by the presence of many neurons showing strong interval and time dependencies (i.e. relatively stereotyped firing patterns), together with an absence of GAD-positive stained cells (putative GABAergic inhibitory neurons: Neale et al. 1983), the proclivity for bursting discharges could be attributable to an early predominance of excitatory synaptic activity in these neocortical cell cultures. A similar developmental sequence appears to occur also in spinal cord cell cultures (Jackson et al. 1982; O'Brien and Fischbach 1986). Excessive positive feedback has also been implicated in the synchronized neuronal bursting activity which characterizes chronically isolated cortex slabs in vivo (Purpura and Housepian 1961). Of course, the possibility cannot be excluded that also extrasynaptic mechanisms, such as intrinsic bursting (Connors et al. 1982) and electrical coupling among adjacent neurons (Connors et al. 1983), contribute to the stereotypy of early firing patterns.

In the intact rat brain, postsynaptic receptor binding for GABA increases dramatically from day 8 to 4 weeks postnatally, correlating well with GAD staining as a presynaptic marker (Coyle and Enna 1976). Since putative GABAergic neurons were observed only from day 15 in the present culture series, the overall decline in interval dependencies after 12 div could be the result of an increase in inhibitory synaptic drive. An overall enhancement of synaptic efficacy, inferred from the rise between 12 and 19 div in the "maturity index" *Hdp/Bdp*, is also a potentially important factor in this development. Finally, a further rise in the numerical density of synapses, plus an increasingly extensive GAD-immunopositive network from three weeks in vitro, coincidences with the appearance of prominent minute-order fluctuations in neuronal firing rates. Similar fluctuations have been observed in immature neuronal networks both in vivo (see Corner, 1985) and in vitro (Corner and Crain 1972; Droge et al. 1986), so that the phenomenon is likely to be a widespread one. Its underlying mechanisms are as yet unclear, but the present findings suggest that a certain degree of maturity, qualitative as well as quantitative, may be necessary for its manifestation.

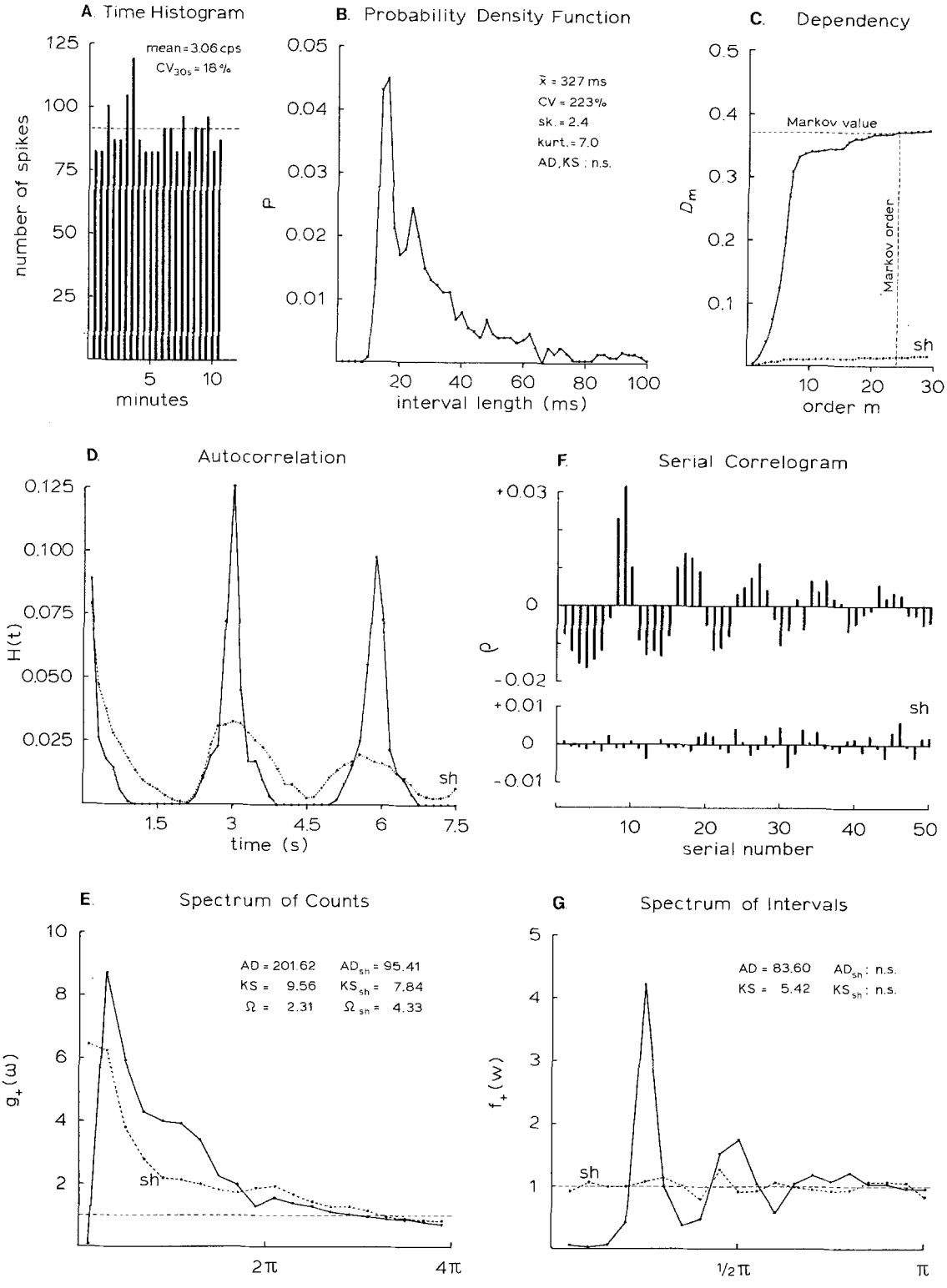


Fig. 3A–G. Spike train analyses exemplified using data from the stereotyped bursting unit depicted in text-Fig. 1A. **A** Time histogram at a bin width of 30 s. **B** Probability density function at a bin width of 5 ms. sk.-skewness; kurt.-kurtosis; **C–G** sh: stands for analyses of the interval series after repeated shuffling. The shuffled series corresponds with a renewal process, which is represented by the broken line in **G**. Converting the shuffled interval series to a time series does not, of course, necessarily result in a sequence of events that corresponds with a Poisson process (broken line in **E**). **E** Maximum in the second class of spectral values corresponds with a mean frequency of 0.46 cps., which is the burst frequency. Horizontal scale ends at 6 cps, which is about twice the mean firing frequency (compare with **A**). **G** Maximum in the fifth class of spectral values corresponds with a mean period of nine intervals (compare with **C**)

Appendix: spike train parameters

I. Parameters computed over stationary samples of successive interspike intervals

In case of more stationary (non-overlapping) samples per record the mean was used. Testing of Poisson- and renewal hypotheses of spike timing and interval generation are discussed in Cox and Lewis (1966) and in De Kwaadsteniet (e.g. expressions 17a–c and 18, 1982). Examples of various analyses are depicted in appendix Fig. 3.

I. 1. Parameters describing the interval distribution (see Fig. 3B)

1.1. Mean interspike interval.
1.2. Coefficient of variation (CV) of the intervals. Defined as the standard deviation divided by the mean.
1.3. Skewness.
1.4. Kurtosis.
1.5. Gamma (reciprocal of the CV, squared). If gamma is less than unity, the distribution represents an exponential decay, whereas if it takes a value of 10, a Gaussian distribution is approached.
1.6. Anderson-Darling statistic (W^2): the 1% significance level is reached for $W^2 = 3.857$, indicating a deviation from an exponential interval distribution.
1.7. Kolmogorov-Smirnov statistic (D): The normalized test value D (Harter 1980) reaches statistical significance (at the 1% level) for $D = 1.63$

I.2. Parameters describing temporal or serial orderliness

2.1. Anderson-Darling statistic, measuring the departure from a flat spectrum-of-counts (i.e., deviation from a Poisson process). Figures 3D, E show, respectively, the autocorrelation function and the corresponding spectrum-of-counts for a non-Poissonian spike train.
2.2. Kolmogorov-Smirnov statistic, also measuring the departure from a flat spectrum-of-counts.
2.3. Omega. Mean spectral value of the spectrum-of-counts, which will be near to unity in the case of a Poisson process.
2.4. Anderson-Darling statistic, measuring the departure from a flat spectrum-of-intervals (i.e., deviation from a renewal process). Figures 1F and 1G show, respectively, the serial correlogram and the corresponding spectrum-of-intervals for a non-renewal spike train.
2.5. Kolmogorov-Smirnov statistic, also measuring the departure from a flat spectrum-of-intervals.
2.6. Markov value. Measures the statistical dependency of the length of an interval on the preceeding interval, as illustrated in Fig. 3C. Higher-order dependencies too can be detected in this way. The Markov value takes a value of zero in the case of no dependency and a value of unity in the case of complete predictability on the basis of the interval history.
2.7. Markov order. Gives the number of preceeding intervals that significantly contribute to the Markov value (see Fig. 3C).

II. Parameters computed over the total spike train record

II.1. 'Bursting' (spike cluster) parameters

Intervals are separated into relatively short ('intraburst') and long ('interburst') intervals, the discriminating interval length being the mean interval for the spike train in question.
1.1. Burst-duration. Mean total time of successive 'short' intervals.
1.2. Burst-period. Mean time from the onset of one burst to the onset of the next one.
1.3. Coefficient of variation of the burst period.
1.4. Burst-ratio. Mean number of successive 'short' intervals divided by the mean number of interburst (i.e., 'long') intervals.
1.5. Burst-intensity. Mean number of successive 'short' intervals divided by the burst-duration (spikes per second).

II.2. Variability in mean firing rate

The Coefficient of Variation was computed for the number of spikes in time bins of differing length: 1, 5, 30, 60 and 120 s (see text-Fig. 1B, appendix-Fig. 3A).

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