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Short Communication

Spontaneous neuronal discharge patterns in developing organotypic mega-co-cultures of neonatal rat cerebral cortex

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ABSTRACT

Sagittal slices of neonatal rat neocortex, extending from the prefrontal to the occipital area, were cultured separately or in pairs, oriented in such a way that axons projecting from the ventricular surface of each explant could innervate the other one. Functional connections were made between as well as within the explants, and spontaneous field potentials and associated action potentials occurred in variable bursts, and with varying degrees of synchrony. Spike-train analysis revealed that the activity patterns seen in these 'mega' co-cultures closely mimic 'tracé alternant' patterns, consisting of trains of burst discharges recurring several times per minute, which are characteristic for the immature intact cerebral cortex during slow-wave sleep. The prefrontal region was consistently less active than the occipital area but the two were qualitatively similar with respect to their patterns of neuronal firing. Isolated mega-cultures, on the other hand, despite their large size, exhibited only intermittent brief bursts that closely resembled those observed both in occipital cortex tissue fragments and in dissociated cell cultures. The mega-co-culture preparation thus appears to give the best currently available approximation to intrinsic cerebral discharge patterns in vivo.

Introduction. Like most other parts of the central nervous system (Ben-Ari, 2001), the cerebral cortex has the property of 'spontaneously' generating neuronal action potentials (SAPs), i.e., in the absence of synaptic input from external sources. This phenomenon is especially pronounced at early stages of development and, using in vitro 'model' systems, has been shown to be an important epigenetic factor (for a comprehensive review see Corner et al., 2002). This intrinsic neuronal activity typically consists of intermittent spiking which is interrupted at variable intervals by relatively intense spike clusters that spread throughout the excitable portions of the cortical network (Jimbo and Robinson, 2000; Robinson et al., 1993; van Pelt et al., 2004). In isolated explant cultures, such 'network bursts' typically last 100–200 ms, and are accompanied by field potentials which resemble the 'K-complexes' seen in the intact cerebral cortex during light slow-wave sleep (Amzica and Steriade, 1997). Periods of a minute or more without any bursts are often seen to alternate with shorter epochs of heightened activity during which network bursts occur at intervals of a few seconds.

In comparison with dissociated cell cultures or isolated explants, pieces of occipital cortex which are co-cultured in close apposition to one another in a ventricle-to-ventricle orientation (Baker and van Pelt, 1997) display high spontaneous firing rates, along with a strong tendency for slow-wave/spike-burst discharges to follow one another in rapid succession (Corner et al., 2005, in press). Since such trains are usually interrupted after several seconds by somewhat longer quies-

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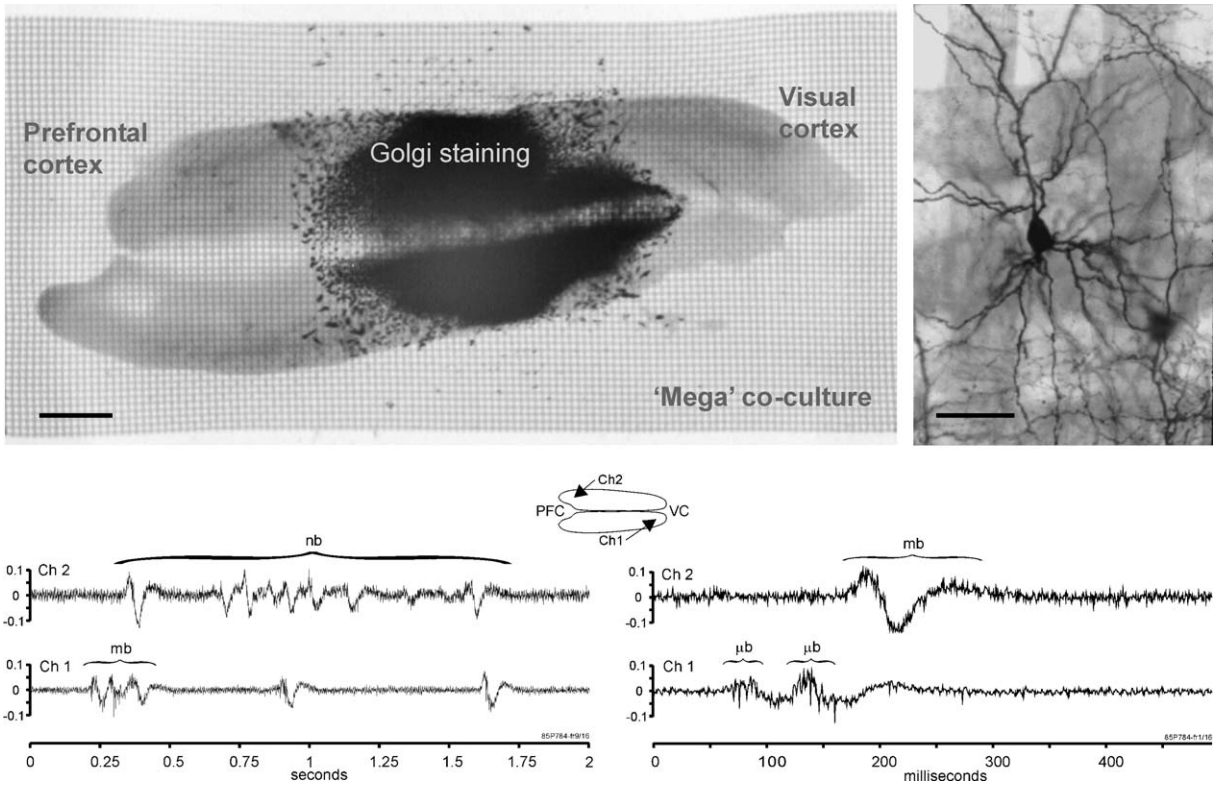

Fig. 1 – Upper panels. Photo-micrographs of (left) two apposed 'mega' explants of neocortex tissue (scale bar: 1 mm), and (right) a Golgi-stained pyramidal cell (scale bar: 50 μm). Lower panels. Examples (at two different time scales) of concomitantly recorded field potentials and spike discharges (filter settings: 10–5000 Hz, 6 dB/octave), showing the tendency for synchronous firing across large distances as well as for such network bursts (nb) to frequently consist of trains of brief spike clusters ('micro-bursts': μb) which themselves often occur in rapid succession ('mini-bursts': mb). VC: visual cortex; PFC: prefrontal cortex.

cent episodes, spontaneous activity in these preparations bears a resemblance to the tracé alternant pattern seen during slow-wave sleep in the intact infant brain (Myers et al., 1997). In co-cultured cortical networks, this firing pattern has proven to be highly resilient, rebounding remarkably during chronic pharmacological suppression of the glutamatergic synaptic receptors which normally mediate most of the excitatory drive (Corner et al., 2005). The conjecture that the existence of such rapidly acting 'homeostatic' mechanisms is in itself suggestive of the importance of spontaneous bioelectric activity for the maturation of neuronal networks has been supported experimentally for a variety of CNS regions (for review, see (Corner

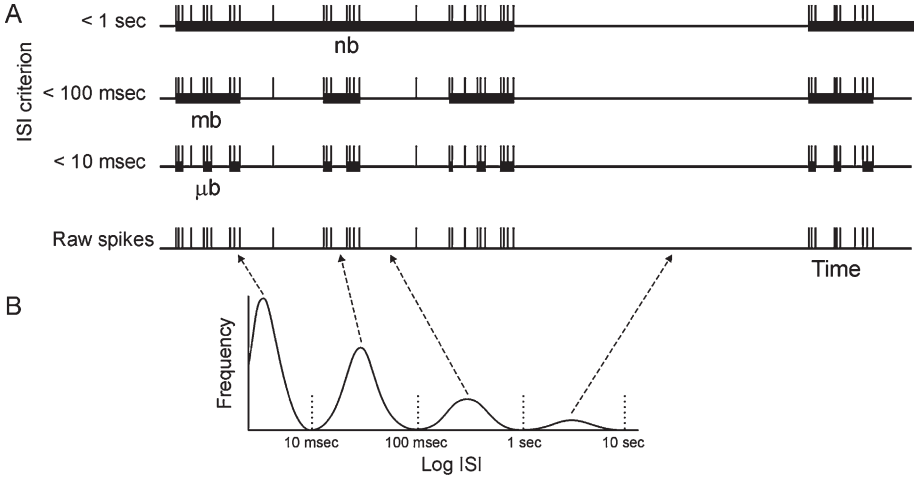

Fig. 2 – Example of application of the interspike interval criterion for defining bursts as sequences of intervals shorter than the criterion value. The multimodality of the idealized interspike interval distribution is used to select the criteria from the minima in the composite ISI distribution.

et al., 2002; Kubitzer and Kahn, 2003; Turrigiano and Nelson, 2004).

Spontaneous firing rates have generally been low compared with in situ measurements: even co-cultured explants exhibit a lower incidence of SAP bursts (Corner et al., 2005, in press) than is seen in the intact rat occipital cortex (Mirmiran and Corner, 1982). The question rises of how to improve the preparation so as to more closely approximate in vivo spontaneous firing patterns. This is an important consideration for any putative 'model system', since recent experiments have indicated that SAP levels play an important part in the development of network excitability (Corner et al., 2002, in press). Since given sites in a cortical network are continually being re-excited by bursts originating elsewhere (Jimbo and Robinson, 2000; Robinson et al., 1993; van Pelt et al., 2004) rather than being solely dependent on intermittent self-stimulation, larger explants might be expected to yield a better approximation to in vivo activity levels. An attempt has therefore been made in the present experiments to more closely mimic the 'tracé alternant' pattern observed in vivo by co-culturing much larger slices of neonatal rat tissue than was the case in earlier experiments. It will be shown that such preparations succeed in combining a more natural neuronal firing mode with reduced intragroup variability. This improved culture system has the additional advantage of allowing simultaneous recordings to be made from widely separated regions of the neocortex (see Fig. 1).

Experimental procedures. The cerebral hemispheres of 5-day-old rat pups were removed, and the neocortex was cut sagittally into thin slices so as to encompass all regions from the prefrontal to the occipital area. Working laterally from the midline, corresponding left and right hemispheric slices were placed on a plastic grid (Romijn et al., 1988) in such a way that intimate physical contact was made between the two components of each preparation, thus enabling nerve fibers

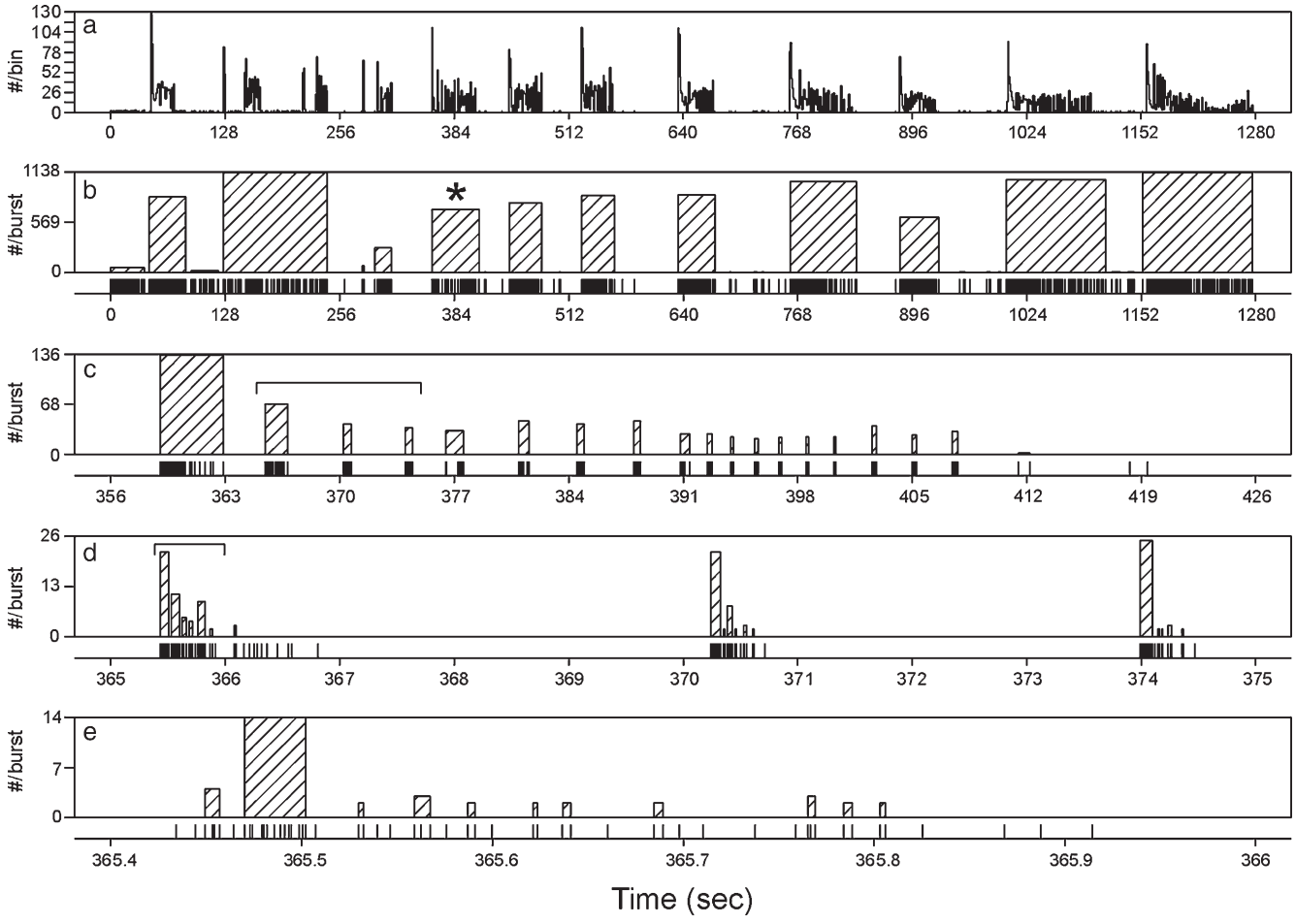

Fig. 3 – Examples of spike clustering and periodicity at several time scales, shown in a firing rate plot of the number of spikes per time bin of 1 s (a), and shown in burst plots defined with spike interval criterion values of, respectively, 5 s (b), 1 s (c), 20 ms (d), and 5 ms (e). The corresponding spike-trains are plotted underneath each burst plot panel. Each burst is characterized by its duration (width of the bar) and number of spikes (height of the bar). Note the different time scales of the panels. Panels a and b illustrate the typical minute-order cyclicity. Panel c zooms in on the spike burst in panel b indicated with a star, and illustrates the typical 'tracé alternant' pattern of network bursts. Panel d expands the three network bursts from panel c indicated with a bracket, and panel e further expands the network bursts from panel d indicated with a bracket. Panels d and e illustrate the tendency for network bursts to be broken up into shorter (mini and micro) spike bursts.

to cross from one side to the other (Baker and van Pelt, 1997). The culture dishes were filled with a specially developed serum-free growth medium (Romijn et al., 1988), placed on a slowly rocking plateau in a humidified incubator at 37 °C, and refreshed at 3–4 day intervals as described earlier for occipital cortex co-cultures (Baker and van Pelt, 1997).

In pilot studies, analog recordings were made of extracellular action potentials and accompanying field potentials at two points simultaneously. These revealed that SAP bursts, which were often synchronous over large distances (Fig. 1), appeared in all regions within a few days after explantation. After 2 weeks of culture, electrodes were positioned under visual control, and extracellular action potentials (high-pass filter: 250–5000 Hz) were recorded for 10–20 min in slowly circulating growth medium. The time-stamps were stored for off-line computer analysis using previously described procedures (Corner et al., 2002, 2005). A single site was monitored on each side of a given explant pair, in a sequence alternating between occipital and prefrontal locations.

Mean firing rates over the entire spike-train were used for first-order statistical comparisons, along with the modal interspike intervals estimated from smoothed semi-log histogram plots (Corner et al., 2002, 2005). In addition to interspike interval criteria of 1000 and 100 ms for defining, respectively, 'network bursts' (because of their synchronous occurrence at different recording sites; (Jimbo and Robinson, 2000; Robinson et al., 1993; van Pelt et al., 2004; and see Fig. 1) and 'mini-bursts' (because these shorter discharges typically appear at several hundred millisecond intervals in the course of a network burst; Corner et al., 2002, 2005), an additional criterion of 10 ms was adopted for defining 'micro-bursts' (so named because of their extreme shortness and tendency to occur repetitively during the mini-bursts: see Figs. 1, 2 and 3). Clustering of spikes on different time scales expresses itself also in a multimodal shape of the interspike interval (ISI) histogram (Corner et al., 2002, 2005). Interval criteria best distinguishing the multiscale clustering in a given spike-train can be estimated from the minima in these ISI histograms (see Fig. 2). Such optimal choices, however, can vary considerably among recording sessions as well as between periods within a session. We have therefore adopted a fixed set of burst criteria, viz. 10, 100 and 1000 ms, since these closely approximate the preferred values found for the majority of preparations. These criteria prove sufficient to reveal the hierarchical clustering of spike discharges on different time scales (see Tables 1, 2 and 3), as well as being adequate for making statistical comparisons between different age and treatment groups (Corner et al., 2002, 2005, in press).

As in our previous electrophysiological studies (see Corner et al., 2002, 2005; Habets et al., 1987), in order to assess the stability of SAP levels from minute to minute, the coefficient-of-variation was calculated over the number of

Table 1 – Comparison between occipital and prefrontal recording sites in 2-week-old neocortical 'mega-explants' at different interspike interval burst criteria

	'Mega' isolates		Occipital co-cultures (25)	'Mega' co-cultures	
	Occipital (21)	Prefrontal (18)		Occipital (13)	Prefrontal (14)
MFR (sp/s)	2.05 (1.17–3.04)	1.29 (0.68–2.07)	5.87 (3.08–7.21) **	7.85 (4.85–9.59) **	4.10 (2.57–7.96) +
Mode (ms)	4 (2–5)	3 (2–4) **	3 (2–6)	8 (7–20) *	7 (6–8) *
BurRatio	0.93 (0.88–0.98)	0.92 (0.84–0.97)	0.99 (0.97–0.99) *	0.95 (0.88–0.96)	0.87 (0.79–0.94) +
CV60	63 (40–82)	55 (45–102) *	69 (50–82)	99 (84–102) **	81 (72–91) +
1000 ms criterion ('network bursts')					
Dur (s)	0.66 (0.19–1.81)	0.44 (0.27–0.84)**	1.78 (0.24–4.45) *	4.67 (2.33–5.11) **	1.52 (1.36–2.09) ++
Int (sp/s)	29.0 (18.2–57.2)	21.4 (11.8–47.1) *	51.8 (35.1–71.3) *	19.0 (14.2–23.5) **	11.5 (9.18–15.0) ++
Per (s/c)	10.9 (8.40–20.0)	10.6 (9.21–20.8) **	10.6 (5.72–26.1)	11.5 (9.60–15.0)	6.16 (5.76–7.35) ++
100 ms criterion ('mini-bursts')					
Dur (ms)	111 (66–211) +	69 (44–88) **	202 (157–290) *	191 (135–240)	135 (120–239)
Int (sp/s)	80.9 (59.4–124)	103 (72.2–125) **	104 (79.4–116)	40.0 (21.3–67.0) **	68.1 (58.2–75.0) ++
Per (ms/c)	437 (402–468) +	399 (384–454) **	456 (414–529)	468 (417–506)	482 (447–540)
10 ms criterion ('micro-bursts')					
Dur (ms)	14 (11–18)	14 (10–19)	16 (13–20)	13 (9–14)	14 (12–17)
Int (sp/s)	275 (251–320)	286 (253–332) **	273 (244–301)	254 (234–279)	246 (234–267)
Per (ms/c)	44 (40–46)	44 (36–50)	45 (42–47)	44 (44–47)	44 (41–46)

MFR: mean firing rate in spikes per second (sp/s); Mode: modal interspike interval in milliseconds (ms); CV60: coefficient of variation (%) over the number of spikes in 60 s time bins; BurRatio: proportion of spikes included within bursts defined by the 100 ms interspike interval criterion; Dur: mean duration of bursts defined by the indicated criterion; Int: mean intensity of intraburst firing in spikes per second; Per: mean time, in seconds or milliseconds per cycle (c), between the onset of successive bursts (see text for the exclusion of large period values). All values are given as the median and (in parentheses) the 50-percentile range for the number of cultures indicated for each group (the data for the occipital co-cultures is taken from Corner et al., 2005).

* $P < 0.05$.

** $P < 0.01$ for the difference between co-cultures and the corresponding isolates.

\+ $P < 0.05$.

++ $P < 0.01$ for the difference between the corresponding prefrontal and occipital 'mega' groups.

Table 2 – Non-parametric 'coefficient-of-variation' (the 50-percentile range divided by the median) at occipital (OCC) and prefrontal (PRE) recording sites in three different types of preparation (see Table 1 for abbreviations and the numerical values on which these calculations are based)

	Mega-isolates		Co-cultures OCC (23)	Mega-co-cultures	
	OCC (19)	PRE (18)		OCC (12)	PRE (9)
MFR (sp/s)	0.91	1.08	0.70	0.60	1.31
CV60	0.67	1.04	0.46	0.18	0.23
1000 ms criterion ('network bursts')					
Dur (s)	2.45	1.30	2.37	0.60	0.48
Int (sp/s)	1.34	1.65	0.70	0.49	0.50
Per (s/c)	1.06	1.09	1.92	0.47	0.29

spikes falling in successive 60 s time bins. Taking advantage of the fact that epochs separating trains of network bursts in neocortex cultures are often largely devoid of action potentials, and that successive network bursts usually occur at intervals of only a few seconds (see Fig. 3), an additional interspike interval criterion of 10 s for burst definition was employed in order to estimate the actual periodicity of 'minute-order' fluctuations in mean firing rates (Corner et al., 2005; and see Figs. 1 and 3).

For each of the above-mentioned interspike interval criteria for defining the occurrence of spike bursts (see Fig. 2), the following parameters were calculated per preparation: (i) mean burst duration, (ii) mean firing rate (intensity) during bursts, and (iii) mean time elapsing between the onset of successive bursts (period). Because the proportion of clustered spikes (i.e., the burst ratio) constantly approached 100% when a criterion of 1000 ms was employed, the ratios listed in Table 1 for statistical purposes are based on the 100 ms criterion. The mean period of bursts obtained for the 100 ms criterion has been calculated for period values smaller than 1000 ms. Likewise, the mean period of bursts obtained for the 10 ms criterion has been obtained for period values smaller than 100 ms. Thus, the mean burst period for a given criterion value denotes burst periodicity within the burst aggregation level obtained for the next higher criterion value. Medians and 50-percentile ranges for each group are presented in the form of tables, which also indicate the results of non-parametric statistical comparisons made using the Chi-square and Mann–Whitney '*U*' tests (two-tailed). For acute treatment effects, Wilcoxon's matched-pairs signed-ranks test was used (Siegel, 1956).

Experimental results. Despite their much larger size, isolated 'mega' explants showed SAP levels and patterning comparable to those previously reported for isolated explants prepared with occipital cortex tissue only (see Corner et al., 2002). Thus, mean firing rates in isolated mega-cultures were considerably lower and the network bursts shorter than in co-cultures of occipital cortex (Table 1). Mini-bursts, too, were relatively short-lasting, though to a lesser degree than for network bursts. In addition, although short bursts of action potentials normally are more 'intense' than longer ones (i.e., have a higher mean intraburst firing rate), both network and mini-burst firing intensities were consistently lower in isolated mega-explants than in occipital co-cultures (Table 1). Micro-burst patterning, on the other hand, was virtually identical in the two groups. Prefrontal areas were slightly less active than occipital recording sites but the burst discharges were otherwise very similar in all respects (see Tables 1 and 2; also Fig. 4).

In striking contrast to the isolates, co-cultured neocortical mega-explants showed a further enhancement of SAP activity over that displayed by co-cultures of occipital cortex only (Table 1). Higher mean firing rates (*MFR*) and enhanced minute-to-minute fluctuations (*CV60*) in this group were accompanied by increased interburst 'background' firing (i.e., lower 'burst ratios') and by an increase in the duration of network bursts (1000 ms criterion). Network bursts in the mega-co-cultures tend to be made up of a long train of brief polyneuronal spike discharges, at intervals of a few hundred milliseconds, occupying almost half of the cycle culminating in the following network burst (Table 1; Fig. 4). It is noteworthy that, in contrast, neither mini- nor micro-burst parameters show any notable differences between the isolated and the co-cultured explants. Employing a 10 s criterion for burst definition, minute-order fluctuations of the mean firing rate were found to consist of an active phase lasting about twice as long in mega-co-cultures as in isolated mega-explants, as well as occupying a much greater fraction of the cycle (Table 3). Network burst parameters as well as minute-order fluctuations (*CV60*) were considerably more

Table 3 – Mean 'minute-order' fluctuations as calculated using a 10 s interspike interval criterion for inclusion of spontaneous action potentials in the active phases of the cycles

	'Mega'-isolates		Occipital co-cultures (23)	'Mega'-co-cultures	
	Occipital (19)	Prefrontal (18)		Occipital (12)	Prefrontal (9)
Dur (s)	18.0 (8.82–29.2) $^{+}$	12.9 (10.5–22.0) **	15.5 (10.2–20.0)	63.5 (35.0–85.6) **	66.2 (48.9–84.2)
Int (sp/s)	4.05 (2.19–5.86) $^{+}$	2.44 (2.01–4.58) *	4.90 (2.42–10.6)	11.9 (4.00–19.7) **	5.27 (4.14–10.6) $^{+}$
Per (s/c)	49.2 (37.0–76.8) $^{+}$	37.2 (35.1–49.5) **	44.0 (32.8–72.2)	94.8 (64.3–142) *	92.6 (81.5–111)

Abbreviations as in Fig. 1. All values are given as the median and the 50-percentile range for the number of cultures given above in parentheses (the data for the occipital co-cultures is taken from Corner et al., 2005).

* $P < 0.05$.

** $P < 0.01$ for the difference between co-cultures and the corresponding isolates.

$^{+}$ $P < 0.05$.

$^{++}$ $P < 0.01$ for the difference between the corresponding prefrontal and occipital 'mega' groups.

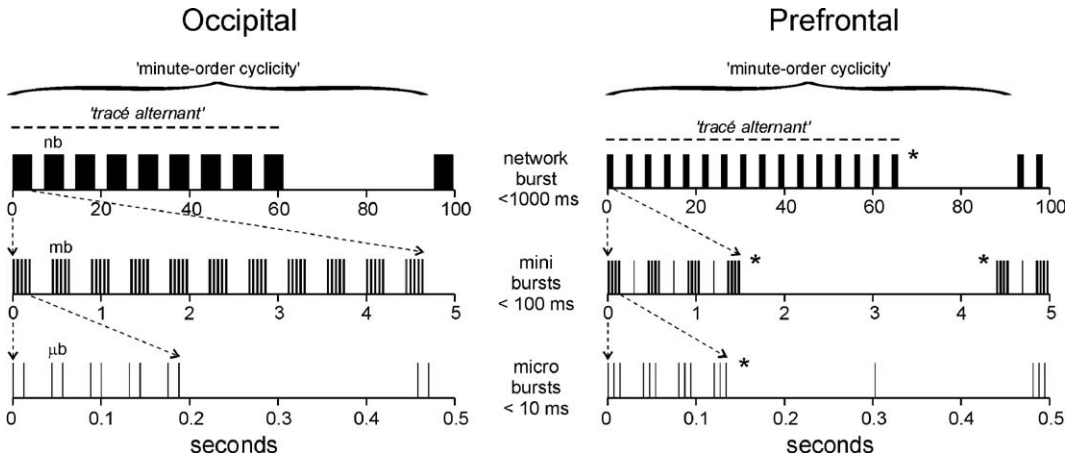

Fig. 4 – Reconstruction of representative average spike-train patterns at, respectively, occipital and prefrontal recording sites in neocortical mega-co-cultures, based on the median values presented in Tables 1 and 3 (bold type) (no attempt has been made to illustrate the quite considerable variations in any given preparation from one burst to the next with respect to duration, intervals, or number of spikes). An asterisk following a spike cluster indicates a statistically significant difference between the two cortical regions with respect to either burst duration or spike density on a particular time scale (see Tables 1 and 3 for exact numerical values); an asterisk preceding a spike cluster indicates a difference in burst periodicity.

consistent from preparation to preparation in the mega-co-cultures than in co-cultures made using occipital cortex only (see Table 2).

Finally, in order to verify the likelihood of cholinergic mechanisms being active also in mega-cultures, as reported for co-cultured occipital cortex explants (Corner et al., 2005), recordings of spontaneous activity were made in the presence of acutely added atropine. The results confirm the existence of an inhibitory cholinergic component in these isolated mega-preparations: greatly augmented burst activity appeared almost immediately upon exposure to the muscarinic receptor blocker (Table 4), against a background of continuous low-level firing which had hitherto been absent (data not shown) and which precluded analysis of spike clustering using the 10 s criterion. It is noteworthy that a clearcut ' burst-pause' pattern, consisting of episodes of intensified spiking every few seconds, was nevertheless still plainly evident in most cases. Even more striking is the persistence of detectable delta- and beta-frequency patterning – i.e., sequences of 'mini'- and 'micro'-bursts, respectively – within these network bursts (see Table 4) despite the potentially confounding effect of the ongoing 'background' firing.

Discussion and conclusions. Since, as was pointed out earlier, firing patterns in developing mega-co-cultures approximate the 'tracé alternant' EEG pattern which characterizes brain activity in sleeping infants (Myers et al., 1997), functional maturation in this new 'model system' gives the closest presently available in vitro approximation to development in situ. In combination with reciprocal cross-innervation (Baker and van Pelt, 1997; Corner et al., 2005), then, network size is indeed capable of making a significant difference for neocortical maturation. These results thus provide further evidence for the intrinsic origin of neocortical activity patterns characteristic for slow-wave sleep (see Lopes da Silva, 1991). The occurrence of alternating epochs of high and low bioelectric activity, on the order of a few minutes, appears to be another general feature of slow-wave sleep in developing homeotherms (Corner and Bot, 1969; Mirmiran and Corner, 1982; O'Brien et al., 1987). Their presence in isolated neuronal networks in vitro suggests that these oscillations, too, are generated within the cerebral cortex itself.

This new 'mega' variant of the co-culture approach has the additional virtue of enabling widely separated regions to be studied simultaneously. In the present experiments, the prefrontal region was consistently less active and its network bursts weaker than for the occipital region, with these

Table 4 – Effect of muscarinic cholinergic blockade on spontaneous spiking parameters in the occipital area of 2-week-old isolated neocortical mega-explants (n = 6)

	Control	+ atropine
MFR (sp/s)*	2.42 (1.84–2.92)	6.67 (5.93–7.44)
Mode (ms)	5 (2–7)	5 (4–6)
CV60*	96 (66–132)	39 (36–42)
BurRatio	0.92 (0.87–0.97)	0.87 (0.78–0.95)
1000 ms criterion		
Dur (s)*	0.76 (0.58–1.17)	2.33 (1.42–6.55)
Int (sp/s)	42.9 (19.7–67.9)	20.5 (8.36–35.8)
Per (s)	10.7 (9.99–13.0)	8.87 (6.25–11.3)
100 ms criterion		
Dur (ms)	195 (159–258)	169 (100–262)
Int (sp/s)	84.6 (77.4–110)	108 (78.6–122)
Per (ms)	483 (453–524)	536 (504–570)
10 ms criterion		
Dur (ms)	19 (15–23)	28 (24–31)
Int (sp/s)	272 (244–309)	268 (248–284)
Per (ms)	39 (36–47)	40 (38–41)

Abbreviations as in Fig. 1. All values are given as the median and the 50-percentile range.
* $P < 0.05$ for the effect of acutely added atropine upon the control cultures (in R16 growth medium).

differences being more pronounced in co-cultures than in isolates. In both types of preparation, moreover, slow fluctuations in the overall firing level were less pronounced in the prefrontal than in the occipital area. Since these parameters are strong indicators of functional development in vivo and, even in the sleeping state, continue to increase for almost three months after birth (Corner and Mirmiran, 1990; Corner et al., 1992), prefrontal cortex appears to ontogenetically lag behind occipital cortex. This is consistent with evidence that neocortical maturation is regionally hierarchical, with primary sensory areas maturing faster in many respects than association areas (for review, see Guillery, 2005). The present results suggest, however, that certain differences are determined already at an early stage and then proceed intrinsically, rather than depending on sequential afferent innervation (Guillery, 2005).

In closing, we wish once again to draw attention to the prevalence within 'network' bursts of faster burst rhythms, especially in the 20–25 Hz ('beta') and 2–3 Hz ('delta') frequency ranges (see Table 1). It is probably no coincidence that beta (~12–25 Hz) along with delta (~1–4 Hz) waves constitute the fundamental components of spontaneous neocortical electrical activity in all warm-blooded animals, and are a persistent feature of all states of vigilance from deep sleep to strong arousal (Bullock and Basar, 1988). These 'bursts within bursts' thus appear to reflect an extremely robust underlying neocortical organizational principle. Whereas beta spike cluster parameters were virtually identical regardless of whether or not a mega-explant was co-cultured alongside a matching piece of neocortical tissue, delta-bursts differed considerably under these two conditions, in particular with respect to the number of mini-bursts comprising a single network burst: 1–2 mini-bursts in the case of isolated explants, ~3–10 for the co-cultures. Here too, the organotypic mega-co-culture neocortex preparation appears to be a greatly improved 'model' for the intrinsic dynamics of cerebral cortex tissue.

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