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Review

Physiological consequences of selective suppression of synaptic transmission in developing cerebral cortical networks *in vitro*: Differential effects on intrinsically generated bioelectric discharges in a living 'model' system for slow-wave sleep activity

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ABSTRACT

Within the context of an updated thorough review of the literature concerning activity-dependent cerebro-cortical development, a survey is made of recent experiments which utilize spontaneous spike-trains as the dependent variable in rodent neocortex cultures when synaptic transmission is interfered with during early ontogeny. Emphasis is placed on the complexity of homeostatic adaptations to reduced as well as intensified firing. Two kinds of adaptation are distinguished: (i) rapid recovery (within several hours) towards baseline levels despite sustained blockade of excitatory synaptic transmission, and (ii) the generation of essentially normal firing patterns in cultures assayed in control medium following development in the presence of excitatory receptor blockers. The former category of homeostatic responses is strongly dependent on the type of preparation, with isolated organotypic explants showing greatly limited plasticity in comparison with co-cultures of matching contralateral pieces of cortical tissue. In such co-cultures, compensatory excitatory drive manifests itself even when all three known types of ionotropic glutamate receptors are chronically blocked, and is then mediated by (muscarinic) cholinergic mechanisms which normally do not contribute measurably to spontaneous activity.

The rapid return of high levels of spontaneous firing during sustained selective glutamatergic receptor blockade appears to protect neuronal cultures treated in this way from becoming hyperexcitable. In particular, quasi-epileptiform paroxysmal bursting upon return to control medium, such as appears in preparations where bioelectric activity has been totally suppressed during network formation, fails to appear in chronically receptor blocked cultures. On the contrary, desensitization of blocked glutamate receptors, as a physiological compensation for the up-regulation of non-blocked receptors, could be demonstrated for both the AMPA and the NMDA glutamate receptor sub-types. This wide range of homeostatic responses underscores the importance of spontaneous neuronal discharges for setting and maintaining an optimal balance between excitatory and inhibitory mechanisms in developing neocortical networks.

Contents

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Abbreviations: APV, DL-2-amino-5-phosphonopentanoic acid (50 μM, Sigma); APN, atropine (5 μM, Sigma); BICUC, bicucculine (200 μM, Sigma); DBE, dihydro-beta-erythroidine hydrobromide (20 μM, Sigma); DEH, dihdro-erythroidine hydrobromide (Sigma); DNQX, 6,7-dinitroquinoxaline-2-3-dione (100 μM, Sigma); LY', LY-382284' (50 μM, courtesy of Eli Lilly & Co., Indianapolis); TTX, tetrodotoxin (1 μM, Sigma).

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1. Theoretical and methodological background

Although intrinsic brain rhythmicity had long before been suspected on the basis of the apparently spontaneous movements of mammalian fetuses (see Gottlieb, 1992), it was not until the middle of the 20th century that – by the clever use of isolated adult amphibian brain/limb preparations (Weiss, 1941,1950) – a central neurophysiological origin for 'non-reflexogenic' motility (Hamburger, 1963) was incontrovertibly demonstrated, and it was not until a decade later that the ubiquity of spontaneous bioelectric activity throughout the developing central nervous system began to be demonstrated (for reviews, see Corner, 1977; Corner et al., 2002; Crain, 1976). With the discovery that rapid-eye-movement sleep, too, represents a striking example of intrinsically generated brain activity, one that persists even through adulthood (Jouvet, 1967), it was not long before the first arguments for its possible ontogenetic significance appeared in the literature (Jouvet, 1967; Roffwarg et al., 1966). Experimental evidence eventually became available that REM sleep indeed contributes significantly to the structural and functional maturation of the brain (reviewed in Corner et al., 1980; Mirmiran, 1995; Vogel et al., 1990; more recently, see Alexandre et al., 2006; Frank et al., 2001, 2006; Martin et al., 2007; Shaffery et al., 2002, 2005).

Despite early reports that spontaneous bursts of post-synaptic action potentials are instrumental in the pruning of exuberant axonal branching that leads to regionally selective innervation in both the spinal cord (Baker, 1985, 1988; Baker et al., 1982, 1984; Corner et al., 1987a,b) and the forebrain visual system (Reiter and Stryker, 1988; Shatz, 1990; Wong, 1993), it was not until the following decade that more than sporadic attention began to be paid to spontaneous, as opposed to sensory evoked, neuronal activity as a dynamic self-regulating 'epigenetic' factor (discussed in Corner, 1994). A veritable explosion of experimental and theoretical investigations since then has made it clear that this hitherto largely neglected factor is actually one of the major guiding principles at all levels of the nervous system (e.g., Ben-Ari, 2001; Silberberg et al., 2005; Spitzer et al., 2002; Peng et al., 2007), in phylogeny as well as ontogeny (comprehensively surveyed in Corner et al., 2002). The vertebrate cerebral cortex must be numbered among these intrinsic bioelectric rhythm generators (Frost and Gol, 1966; Jouvet, 1967; Kellaway et al., 1966; Steriade et al., 1990, 1993; Timofeev and Steriade, 1996), and recent studies of its wide range of activity-dependent properties will be summarized in the present review.

Neuronal firing of intrinsic origin (i.e., 'spontaneous' action potentials: SAPs) is now well established as being an indispensible factor for the fine-tuning of neural networks throughout the developing central nervous system (CNS), ranging from the selective innervation of target cells to the balance between excitatory and inhibitory synaptic drive (see Corner et al., 2002 for an earlier survey; further, Ben-Ari, 2006a; Cohen-Cory, 2002; Karmarker and Buonomano, 2006; Moody and Bosma, 2005; Spitzer, 2006; Zhang and Poo, 2001). SAP-dependent maturation has recently been documented for a variety of central nervous regions, including the *corpus striatum* (Gustafson et al., 2006), *hypothalamus* (Belousov et al., 2002, 2004), *retina* (Butts et al., 2007; Grzywacz and Sernagor, 2000; Harris et al., 2002), *superior colliculus* (Chandrasekaran et al., 2007; Meier et al., 2002, 2003; Razak and Pallas, 2007), *lower brainstem* (Kotak and Sanes, 2003), *cerebellum* (Andjus et al., 2003; Liu and Cull-Candy, 2002; Nakanishi and Okazawa, 2006; Ouardouz and Sastry, 2005) and *spinal cord* (Galante et al., 2001; Rosato-Siri et al., 2002) but the present updated survey – which is complete in principle through December 2007 – will be essentially restricted to the cerebral (paleo- and archi- as well as neo-) cortex.

Activity dependence in the earliest stages of functional development (the '*premature*' phase of neuroplasticity: Corner et al., 2002) typically takes the form of a homeostatic response whereby the network compensates for abnormal increases or decreases in ongoing activity (for additional recent development of this idea, see Alexandre et al., 2006; Belousov et al., 2001; Borodinsky et al., 2004; Burrone et al., 2002; Burrone and Murthy, 2003; Chandrasekaran et al., 2007; Corner et al., 2005, 2006; Davis and Bezprozvanny, 2001; Kubitzer and Kahn, 2003; Marder and Goaillard, 2006; Pratt and Aizenman, 2007; Spitzer et al., 2004, 2005; Turrigiano and Nelson, 2000, 2004). In this way, spontaneous firing in the developing brain would serve as an indicator of overall network excitability, with levels above or below some preset range – the ontogenetic origins and of which are presently unknown – leading to down- or up-regulation, respectively, of the mechanisms responsible for maintaining phylogenetically selected, presumably 'optimally' programmed, levels of neuronal responsiveness.

The earliest homeostatic neurophysiological mechanism to be discovered was the direct dependence of inhibitory maturation in the cerebral neocortex on spontaneous firing levels (Corner and Ramakers, 1992; Ramakers et al., 1990); other factors – in particular, the sensitivity of excitatory glutamatergic receptors – were subsequently found to be involved as well (reviewed in Corner et al., 2002). Indeed, the plethora of cortical processes which have been demonstrated in recent years to be sensitive to changes in ongoing bioelectric activity (see Section 5.6; also

Ben-Ari, 2006a; Ben-Ari and Holmes, 2006; Moody and Bosma, 2005; Spitzer, 2006) is in itself strong putative evidence for the importance of such activity for normal development. At the same time, this is a form of plasticity that could become problematic if functional disturbances occur during a 'sensitive phase' of development, e.g., during intrauterine life, after which the brain becomes exposed to normal physiological conditions. The earlier adaptations could then lead to a more or less permanent hypo- or hyperactivity that might prove to be extremely difficult to correct later on (De Lima et al., 2004; Houweling et al., 2005; Friel and Martin, 2005; Van Huizen et al., 1987a; Van Oss and van Ooyen, 1997).

Quantitative dendritic measurements have established that cyto-morphological development is very poor in isolated organotypic neocortex explants despite the retention of a characteristic histotypic organization (Baker and van Pelt, 1997). In addition, SAP firing patterns in such explants show less change with maturation in culture than do dissociated cell cultures (cf. Corner and Ramakers, 1992; Ramakers et al., 1990). With the discovery, then, that when two cortical slabs – especially if relatively large in size (Baker et al., 2006) – are co-cultured in close apposition so as to enable reciprocal innervation, firing levels as well as dendritic development begin to closely approximate the *in vivo* picture (Baker and van Pelt, 1997; Corner et al., 2005, 2006), an improved preparation became available for checking the reported ontogenetic effects of partial or complete synaptic receptor blockade on the development of SAP discharge patterns (see Corner et al., 2002). In addition, layer-specific afferent and efferent connections resembling those *in vivo* have been reported for both cortico-cortical and cortico-thalamic co-cultures (Bolz, 1994; Dammerman et al., 2000b; Lotto et al., 1999). Organotypic co-cultures have thus become a preparation of choice whenever maximal relevance to the *in vivo* condition in an *in vitro* 'model' system is required.

In contrast to isolated organotypic explants (Corner et al., 2002), where isolated short bursts (analogous to 'K-complexes' seen during light quiet sleep in the intact brain: Roth et al., 1956; Steriade, 1997, 2006) typically occur every few seconds or at longer intervals, co-cultured explants show a developmental trend towards longer and longer trains of these 'mini'-bursts. Each such burst of activity lasts no more than a few hundred milliseconds, and they follow one another at a mean but variable frequency of 2–3 Hz (thus constituting a train of 'delta/theta waves': Lopes da Silva, 1991; Steriade et al., 1990). Each train lasts several seconds at the most (Baker et al., 2006; Corner et al., 2005, 2006; Seamans et al., 2003) before a variable silent interval of at least 1 s intervenes, thus corresponding to what in the intact neocortex has been called the 'slow' rhythm (Steriade, 1997, 2006; also Picken-Bahrey and Moody, 2003). Individual mini-bursts, in turn, tend to be broken up into a sequence of extremely brief – ca. 10–15 ms – '*micro*'-bursts (Blackwell et al., 2003; Chiappalone et al., 2006; Corner et al., 2005, 2006;) which have an average repetition rate falling in the 'beta/gamma' frequency range, i.e., ~20–50 Hz. (Lopes da Silva, 1991; Steriade et al., 1990). Due to the failure of action potentials to consistently follow high frequency membrane oscillations, the even more rapid "sharp-wave ripples" and "very-fast oscillations" in local cortical field potentials (Brunel and Wang, 2003; D'Antuono et al., 2005; Geisler et al., 2005; Li et al., 2007b; Traub et al., 2005) and the "intrinsic burster" category of neurons (Connors and Gutnick, 1990; Llinas, 1988) would add yet another – '*pico*-burst' – level to the known hierarchy of intrinsic rhythmical network properties.

Since multiple electrode recordings have revealed that even *mini*-bursts can be spatially quite extensive and involve widespread neuronal activation (e.g., Chiu and Welicky, 2001; Van Pelt et al., 2004a,b, 2005), the terms 'network *spikes*' (Eytan and Marom, 2006) and 'population bursts' (Giugliano et al., 2004; Wagenaar et al., 2005, 2006a,b) have also been used in the literature for this species of spontaneous bioelectric activity. We therefore propose, in order to emphasize the usually nested character of the above-mentioned hierarchy of burst durations and see Fig. 1, to substitute the term '*midi*'-bursts for what we used to call 'network bursts' of spike activity (e.g., Corner et al., 2002, 2005; Corner and Crain, 1965; Habets et al., 1987; Ramakers et al., 1990, 1991). [The *in situ* equivalent to these *in vitro* 'midi-bursts' is *phasic* discharges, a term that has often been used (e.g., Corner, 1977, 1990; Jouvet, 1972; Kohyama, 1996; Orem, 1996) to describe spontaneous events lasting on the order of a few seconds, in particular those occurring during episodes of rapid-eye-movement sleep (also see Sallinen et al., 1996; Takahara et al., 2002; Wehrle et al., 2007).]

Burst durations and other parameters, such as frequency of occurrence, spike counts and firing rate profiles, tend to be highly variable on all time-scales (Arieli et al., 1996; Baker et al., 2006; Chao et al., 2005; Corner et al., 2002, 2005; Echevarria and Albus, 2000; Harsch and Robinson, 2000; Madhavan et al., 2007; Wagenaar et al., 2006a), with coefficients of variation seldom less than 0.5 and sometimes exceeding 3.0 for a given spike train. These variations often appear to be highly ordered but this impression has not been subjected to rigorous analysis. Older cultures show the expected increase both in the 'burst ratio', i.e., the proportion of spikes falling between successive mini-bursts, and in the magnitude of minute-to-minute fluctuations in the mean firing rate as estimated by the coefficient of variation over 60 s time-bins (see Corner et al., 2005; Corner and Ramakers, 1992). A cycle of ca. 30 s of heightened spontaneous burst activity every 1–3 min, on the average, is commonly observed in isolated and co-cultured cortical explants (see Corner et al., 2005; Wagenaar et al., 2006a) as well as in acute slices (Le Bé and Markram, 2006) and *in situ* (Corner and Bour, 1984; Corner and Mirmiran, 1990). Since this is the order of 'burst' magnitude, c.q., *active phase*, defined in the original reports on 'non-reflexogenous' (Weiss, 1941) neuro/behavioral

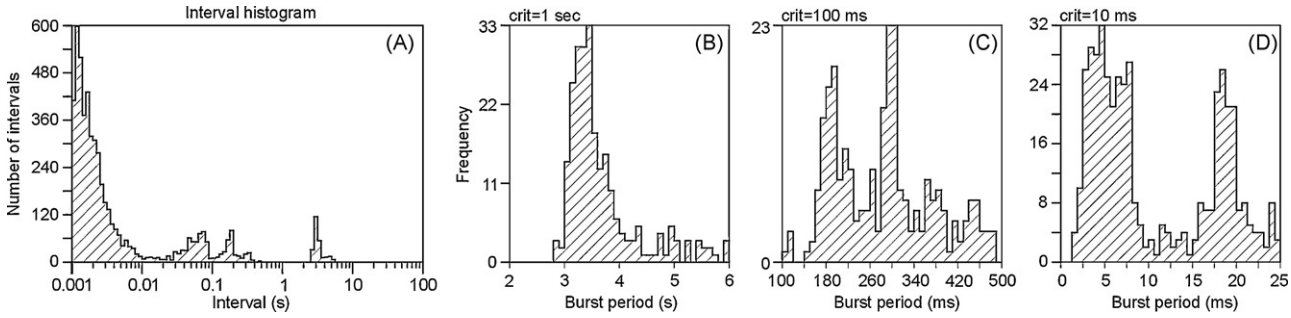

Fig. 1. Burst periodicity histograms in a representative highly active culture: (A) interval histogram showing spike clustering in the four burst ranges defined in Section 5.2; (B–D) burst-period histograms (i.e., intervals between successive burst onsets) for interspike interval criteria of 1 s, 100 ms and 10 ms, respectively.

rhythmicity in chick embryos and anuran larvae (Corner, 1964b; Hamburger, 1963), we propose for the sake of consistency to use the term '*maxi*-burst' (also recently called 'super-bursts': Wagenaar et al., 2006a,b) for the active phase of these 'infra-slow' oscillations within the central nervous system (Aladjalova, 1957, 1964).

Activity fluctuations on still longer time-scales – ranging from >5 min to <24 h – can then be regarded as falling into the broad spectrum of *ultradian rhythms* (see Cook, 1991; Corner, 1977, 1990; Lavie and Kripke, 1981; Le Bon et al., 2007), a range of frequencies which will not be treated in the present review. The designation '*mega*-bursts', incidentally, can best be reserved for 'paroxysmal' population discharges – i.e., highly exaggerated network bursts in terms of either the intensity of firing, the spatial extent of activation, or the density of recruited neurons (Crain, 1976; Wells et al., 2000) – such as sometimes occur spontaneously (Wagenaar et al., 2006a,b; Madhavan et al., 2007; Vajda and van Pelt, unpublished observations), but more often under patho-physiological conditions (e.g., Ben-Ari, 2006a,b; Ben-Ari and Holmes, 2006; Bosman et al., 2005).

2. Homeostatic restoration of spontaneous neuronal firing

2.1. Partial excitatory amino acid receptor blockade

Selective AMPA receptor blockade in isolated occipital cortex explants (using DNQX) induces a slow decline in SAPs, whereas NMDA receptor blockade (using APV) causes only a transient suppression (Corner et al., 2002). Thus, after an initially profound depression, firing levels in 1- and 2-week-old cultures begin to rise within a few hours after exposure to APV, and by 24 h approximate (except for slightly shorter midi-bursts) all of the control values. Acute exposure at this point to DNQX has established that this homeostatically restored activity is completely AMPA receptor driven. At 3 wiv (weeks *in vitro*), in contrast, SAP activity persists at a very low level throughout the entire 24-h period of selective NMDA receptor blockade, and returns to control levels only upon return to control medium (Corner et al., 2002). In order to suppress SAP firing throughout several weeks in isolated cortex explants by means of glutamate receptor blockade, a combination of NMDA and AMPA antagonists is required.

In *co-cultured* cortex explants, on the other hand, even a combined chronic blockade of NMDA and AMPA receptors fails to prevent the appearance of SAPs, the firing levels rising to about half that of control cultures during 2-weeks of culture *in vitro* (Corner et al., 2005). SAP recovery in the growth medium took place to a similar degree even after a mere 24-h exposure to APV + DNQX (Table 1; and Corner, 2008). Using a selective kainate receptor blocker ('LY': Bleakman and Lodge, 1998), it could be established that it is this latter sub-type of glutamatergic receptor which, despite its inability to sustain much SAP activity in control explants, provides the excitatory synaptic drive in chronically or overnight APV + DNQX treated cortical co-cultures (Corner et al., 2005). In the case of selective chronic suppression of either the AMPA or the NMDA glutamate receptor sub-type, on the other hand, full SAP recovery had been completed already within 1-week *in vitro* (although it was noted that the temporal patterning of burst discharges while in the respective growth media deviates in characteristic ways from the SAP patterns seen in control co-cultures). In both cases, up-regulation of kainate receptors was found to have contributed to functional recovery (Corner et al., 2005).

In 1–2-week-old cultures, both AMPA and NMDA receptors initially up-regulate during APV + DNQX treatment to the point where, within 24 h, each can sustain normal firing levels and burst durations on its own (Table 2). With both receptor types unblocked (i.e., upon return to normal growth medium), firing rates greatly exceeded those ever seen in untreated co-cultures: 3.54–**11.0**–17.8 spikes per second (Corner, 2008). Purely AMPA receptor driven networks, however, show inordinately long mini- as well as midi-bursts after 24 h of A + D blockade (Table 2: *100 ms* and *1000 ms* criteria, respectively), whereas in NMDA receptor driven explants it is the only the latter which are significantly longer. Micro-burst durations tend to be relatively long, and intra-burst firing rates correspondingly low, in both groups of co-cultures (Table 2: *10 ms* criterion). In isolated cortical explants, in contrast, overnight adaptation is limited to the AMPA receptors (Corner et al., 2002), a restriction that could be due to a dearth of NMDA receptors on the abortive dendrites which are characteristic for that type of preparation (Baker and van Pelt, 1997).

Table 1
Effects of 24 h exposure to APV + DNQX (A + D) on spontaneous firing patterns in 1-week-old occipital cortex co-cultures recorded in the presence of A + D (the control group was recorded in normal growth medium without added A + D)

	Control (n = 10)	APV + DNQX (n = 11)
MFR (sp/s)**	2.43–**3.26**–5.87	0.99–**1.27**–2.78
Modal ISI (ms)**	15–**30**–150	150–**200**–625
Burst ratio (−/−)**	0.71–**0.85**–0.87	0.24–**0.46**–0.57
CV-60 s (%)*	73–**108**–115	64–**70**–87
1000 ms criterion		
Duration (s)	1.34–**1.91**–2.88	1.43–**1.86**–4.59
Intensity (sp/s)**	8.00–**12.0**–16.0	2.71–**3.51**–4.91
Period (s/c)	5.77–**6.31**–7.52	6.08–**7.49**–15.9
100 ms criterion		
Duration (ms)**	118–**152**–216	49–**65**–83
Intensity (sp/s)**	45.0–**57.6**–68.1	21.4–**25.6**–27.7
Period (ms/c)	422–**451**–479	443–**457**–485
10 ms criterion		
Duration (ms)**	7–**9**–11	4–**5**–5
Intensity (sp/s)**	283–**297**–330	202–**217**–243
Period (ms/c)*	38–**40**–41	46–**52**–66

MFR: mean firing rate; ISI: interspike interval; CV: coefficient of variation (%); sp: spikes; bur: burst; c: cycle; ms: milliseconds; s: seconds. All values are given as the *median* (in bold-face) and the *50-percentile* values for the number of cultures given in parentheses.
*$p < 0.05$; **$p < 0.01$ for the difference between the two groups.

It is noteworthy that, despite large differences in the level of spontaneous firing, the mean periodicities of '*micro*-, *mini*- and *midi*-network bursts' hardly differ from one treatment group to another. A possible explanation is that a lesser degree of refractoriness following weak bursts in relatively quiet cultures is compensated for by sluggishness of the network for bursts to be triggered in the first place, and vice-versa for relatively intense bursting preparations. The extent, however, to which burst rhythms on different time-scales are in fact determined by 'relaxation oscillations' – based upon, for instance, outward membrane current kinetics (for discussion, see Corner et al., 2002) – or, instead, are generated by intrinsic timing mechanisms analogous to the circadian 'clock' (see Scheper et al., 1999) remains to be established.

2.2. Total excitatory amino acid receptor blockade

Even when all glutamatergically driven activity is prevented from appearing by means of a growth medium containing a cocktail ('LAD') of selective blockers of all three glutamate receptor sub-types, spontaneous burst firing in occipital cortex co-cultures nevertheless appears already by 1-week *in vitro* and, although falling short of control firing levels, persists for at least two more

Table 2
Spontaneous firing patterns in 2-week-old occipital cortex co-cultures recorded in control growth medium plus added LY and either APV ('AMPA' group) or DNQX ('NMDA' group) after a 24-h exposure to APV + DNQX ('A/D' groups)

	'*AMPA*': recorded in LY + APV		'*NMDA*': recorded in LY + DNQX	
	Control (6)	24-h A/D (8)	Control (8)	24-h A/D (9)
MFR (sp/s)	0.54–**0.97**–1.88	3.93–**5.87**–8.84*	0.69–**1.44**–3.58	3.20–**3.58**–7.88*
Modal ISI (ms)	400–**850**–1500	2–**50**–250*	30–**70**–400	7–**15**–90
Burst ratio	0.37–**0.44**–0.68	0.58–**0.76**–0.85*	0.64–**0.73**–0.75	0.82–**0.84**–0.85*
CV-60 s (%)	92–**102**–107	65–**97**–225	98–**119**–148	95–**148**–184
1000 ms criterion				
Duration (s)	0.79–**0.99**–1.33	2.74–**5.48**–8.77**	1.33–**1.47**–2.57	2.33–**2.78**–4.20*
Intensity (sp/s)	3.99–**4.85**–12.5	6.59–**9.08**–12.7	7.47–**9.10**–9.92	10.4–**12.5**–13.9*
Period (s/c)	8.90–**11.2**–13.1	7.91–**8.94**–11.1	7.70–**9.67**–18.9	7.50–**11.6**–12.8
100 ms criterion				
Duration (ms)	67–**76**–113	95–**132**–167*	107–**135**–172	129–**133**–173
Intensity (sp/s)	52.0–**63.0**–80.0	48.2–**80.0**–92.4	51.6–**53.1**–85.8	40.1–**53.8**–88.8
Period (ms/c)	408–**464**–571	510–**528**–540	427–**448**–482	420–**426**–444
10 ms criterion				
Duration (ms)	4–**6**–7	6–**8**–12*	8–**9**–9	9–**12**–19*
Intensity (sp/s)	506–**562**–626	287–**410**–546*	311–**404**–451	259–**277**–316*
Period (ms/c)	37–**39**–42	41–**41**–43	30–**34**–37	39–**42**–45*

Abbreviations as in Table 1.
*$p < 0.05$, **$p < 0.01$ vis à vis the corresponding control group.

weeks (Corner et al., 2005). It could be shown by acute APN administration that most of the excitatory drive in such preparations has become cholinergic (largely or exclusively muscarinic, since the selective nicotinic receptor blocker DEH had no noticeable effect on spontaneous spiking: Corner et al., 2005). There also appears to be a smaller contribution of unknown origin, possibly from cortical peptidergic neurons (Goetz and Bolz, 1994). These experiments reveal, furthermore, that intrinsic cholinergic drive (see Houser et al., 1985; Schambra et al., 1989) within organotypic neocortex explants has an inhibitory as well as an excitatory component. Thus, the APN-induced disappearance of network burst activity from chronic LAD-treated cultures is accompanied by the appearance of persistent low-level background firing. Similar disinhibition is induced by atropine in control cultures, too, but in this case (i.e., in the presence of still functioning excitatory amino acid receptors) intensified 'midi'-bursts can be seen superimposed upon the 'tonic' background spike activity (Corner et al., 2005).

Even chronic addition of APN to the 'LAD' growth medium for 2-weeks ('LADA' group) fails to prevent the developmental appearance of SAPs, although at a lower frequency than in control cultures (Corner et al., 2005; and Table 10). Network bursts become relatively short and less intense on all time-scales under these conditions but their incidence is correspondingly higher, while minute-to-minute fluctuations are less pronounced. This homeostatically 'rescued' spontaneous activity cannot be eliminated by acutely blocking the remaining (nicotinic) cholinergic receptors with DBE. If anything, chronic LADA-treated cultures ($n = 12$) become more active following such treatment, exhibiting twofold higher mean firing rates as well as much longer network bursts (ca. 6 s, on the average, vs. 2.5 s in the controls: data not shown). At the same time, such bursts become much more frequent, though less intense, which causes a shift of modal interspike intervals from ca. 5 to 200 ms. Additional signs of the DBE-induced shift from burst to tonic firing include a greatly reduced burst ratio (from ~0.85 to 0.060) and smaller minute-to-minute fluctuations (CV-60 s from ~90% to 55%). Control cultures ($n = 20$) were hardly affected by acute DBE application, suggesting the possibility of up-regulation in an undisclosed intrinsic cortical excitatory system under nicotinic inhibitory control.

2.3. Inhibitory amino acid receptor blockade

Both in dissociated cell cultures (see Corner et al., 2002) and in organotypic co-cultured explants (Corner et al., 2005) chronic blockade of GABAa receptors – using picrotoxin and bicucculine, respectively – induces persistent stereotyped bursting (resembling interictal epileptiform discharges) throughout at least 3 weeks of culture *in vitro*. Such disinhibition thus causes 'freezing' of spontaneous firing in a pattern resembling that seen at the very onset of widespread intermittent bioelectric activity in developing neocortical networks (see Habets et al., 1987; Ramakers et al., 1990) as well as in most other regions of the central nervous system (Ben-Ari, 2001; Corner et al., 2002). Both in primary cell cultures and in organotypic co-cultures the mean firing rate is at first considerably higher in chronically GABA blocked than in control preparations, but gradually falls to lower than control levels over a period of several days (Corner et al., 2005; Turrigiano and Nelson, 2004).

3. Protective effects of homeostatically restored neuronal firing

3.1. Chronic selective glutamate receptor blockade

An extensive study employing organotypic occipital cortex slices cultured in isolation demonstrated that excessive spontaneous spiking occurs upon transfer to control medium following prolonged SAP suppression during the early stages of neuronal network formation (by means of simultaneously blockading AMPA and NMDA receptors using a 'cocktail' of DNQX plus APV: Corner et al., 2002). The fact that similar abnormalities are observed in older explants during acute GABAergic disinhibition suggests that, as in dissociated neocortex cultures (see Corner and Ramakers, 1992; Ramakers et al., 1994), inhibitory synaptic maturation is especially impaired in the absence of SAPs. It became clear from parallel experiments that even the rapid return of spiking to control levels *in vitro*, when NMDA receptors are selectively blocked, is unable to prevent a similar 'hyper-excitability syndrome' from appearing upon transfer to control medium (Corner et al., 2002). However, since control firing levels in 'isolated-explant' preparations are typically much lower than in

Table 3
Spontaneous firing patterns as assayed in control *recording* medium (DMEM) following 2-weeks of culture in the presence of selective amino acid receptor blockers

	Group				
	Control (19)	BICUC (15)	APV (21)	DNQX (18)	APV + DNQX (12)
MFR (sp/s)^	1.19–**2.27**–4.93	1.05–**2.28**–3.90	1.42–**2.31**–2.86	2.46–**4.29**–6.64$^{*\#\prime}$	0.99–**1.93**–7.40^{+}
Modal ISI (ms)^	5–**6**–7	10–**35**–100**	3–**5**–25$^{\#}$	4–**8**–38$^{*\#}$	8–**23**–70*
Burst ratio^	0.89–**0.96**–0.97	0.65–**0.80**–0.95**	0.91–**0.97**–0.98$^{\#}$	0.86–**0.90**–0.97^{+}	+0.67–**0.73**–0.94$^{*\prime\prime}$
CV-60 s (%)^	35–**67**–85	91–**99**–120*	44–71–126$^{\#}$	65–**88**–106^{+}	+92–**120**–78$^{**\#\prime}$
1000 ms criterion					
Duration (s)^	0.76–**2.23**–4.61	1.33–**2.40**–3.02**	1.84–**3.09**–5.18$^{\#\#}$	1.89–**2.54**–3.88	1.84–**3.44**–5.79$^{\#}$
Intensity (sp/s)^	9.76–**17.1**–34.9	7.98–**13.1**–32.7*	8.86–**16.9**–29.4	14.5–**17.8**–23.3$^{*\#\#}$	8.08–**10.3**–17.4^{*+}
Period (s/c)^	7.96–**12.5**–27.5	6.43–**8.90**–12.9**	12.3–**18.6**–28.3$^{\#}$	9.81–**11.5**–16.4$^{*\prime}$	9.20–**18.0**–21.4$^{\#+}$
100 ms criterion					
Duration (ms)^	37–**67**–131	75–**117**–129*	28–**67**–178$^{\#\#}$	100–**154**–192$^{**\#\prime\prime}$	109–**158**–240$^{*\#\prime\prime}$
Intensity (sp/s)^	73.1–**79.8**–91.6	27.7–**49.3**–66.1**	73.8–**88.0**–109$^{\#\#}$	40.4–**66.1**–83.1$^{*\#\prime}$	28.8–**30.5**–55.2$^{**\prime\prime+}$
Period (ms/c)^	464–**553**–627	435–**451**–486**	455–**530**–610$^{\#\#}$	426–**465**–541*	422–**442**–471$^{*\prime}$
10 ms criterion					
Duration (ms)	10–**13**–15	6–**10**–10*	11–**15**–17$^{\#}$	10–**13**–17$^{\#}$	7–**9**–15
Intensity (sp/s)	196–**224**–241	192–**212**–227	217–**230**–270$^{\#}$	198–**238**–272$^{\#}$	188–**204**–220$^{\prime+}$
Period (ms/c)^	31–**39**–45	44–**47**–55*	33–**42**–46$^{\#\#}$	41–**43**–46$^{\#}$	42–**43**–47

Abbreviations as in Table 1.
^Overall *treatment* effect ($p < 0.05$).
$^{*}p < 0.05$, $^{**}p < 0.01$ vis-à-vis the control group.
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vis-à-vis the bicuculline group.
$^{\prime}p < 0.05$, $^{\prime\prime}p < 0.01$ between the APV and the DNQX or the APV+DNQX group.
$^{+}p < 0.05$ between the DNQX and the APV+DNQX groups.

the intact rat neocortex (Mirmiran and Corner, 1982), the possibility remains that SAPs would be able to promote normal physiological maturation even under conditions of NMDA receptor blockade if the homeostatically restored spike activity was intense enough to offset the loss of calcium entry via NMDA-linked channels.

In line with such a possibility, it has been reported that the NMDA receptor dependence for stimulus-induced plasticity in spinal cord neurons can indeed be overcome in APV-treated cultures by tripling the extracellular calcium concentration during electrical stimulation (Fields et al., 1991; Fields and Nelson, 1991). Organotypic hippocampal cultures grown for several days with chronically blocked NMDA receptors indeed appear to develop quite normally, showing only a mild increase in spontaneous inhibitory (and a concomitant decrease in excitatory) post-synaptic potentials, in contrast to the intense paroxysmal discharges measured in TTX-treated sister cultures (Bausch et al., 2006). Since bioelectric activity was not monitored during the treatment period, however, this partial protection against becoming hyperactive upon return to normal growth conditions cannot be unequivocally attributed to restored neuronal firing in the APV-treated preparations.

Co-cultured organotypic explants have elevated SAP firing rates in glutamate receptor blocked as well as in control preparations (see Section 2; and Corner et al., 2006) and, indeed, chronic NMDA receptor blockade in such preparations has not been found to cause any significant deviations from control values when assayed in normal medium (see Table 3: APV group). Chronically AMPA receptor-blocked explants, on the other hand, show a slight but significant hyperactivity upon transfer to control medium after 2 wiv: trains of bursts last longer on all time-scales than in control cultures (see Table 3: DNQX group). Combined treatment with APV and DNQX for 2 wiv exaggerates this effect at both the network and the mini-burst level. On the other hand, it also produces signs of accelerated maturation, viz., greatly reduced burst ratios and longer interspike intervals (Table 3) along with larger minute-to-minute activity fluctuations (also see Table 5: APV + DNQX group). In addition, mini-burst variability is significantly exaggerated: coefficients of variation (for *burst durations*) exceed 2.0 in the majority of cases, as compared with values between 1.0 and 1.5 for almost all preparations in the other groups (data not shown). None of these effects was associated with abnormal SAP levels or patterns during the treatment period (Corner et al., 2005).

By 3 wiv, when recorded in control medium, most of the differences between control and chronically APV + DNQX-treated co-cultures have lessened, with both groups showing a weaker tendency towards burst firing than at 2 wiv (Corner et al., 2006; and Table 4). On the other hand, network burst trains in the experimental group tend to last much longer now (Table 5) while minute-to-minute fluctuations are greatly reduced so that, in this latter respect, there appears to have been a regression to earlier control values (Table 4: CV-60 s). Also the mini-burst durations have reverted to greater stereotypy at 3 wiv – with coefficients of variation now between 0.5 and 1.0 for most explants – whereas control values are still much the same as in younger cultures (data not shown). Network bursts, too, are significantly less variable than in 3-week-old control cultures, although not to the same degree as for the mini-bursts. None of these later developments in the A + D-treated group appear to be attributable to differences in SAP activity during the extended treatment period (see Corner et al., 2006).

The restoration of close to normal SAP patterns in all of the experimental groups during the treatment period constitutes a putative basis for the observed protection of co-cultured cortical networks from becoming hyperexcitable despite the profound prolonged interference with synaptic transmission. The fact that chronic exposure to APV + DNQX, despite the rapid up-regulation of acutely blocked AMPA and NMDA receptors (thus resulting in greatly intensified SBA upon return to normal medium after 24 h: see Section 2 and Corner, 2008), had an unexpectedly mild effect on SAP discharges when assayed in control medium after 2–3 wiv suggests that kainate-driven neuronal firing during the treatment period (see Corner et al., 2005) homeostatically opposes up-regulatory tendencies of other excitatory mechanisms. Indeed, the NMDA and AMPA glutamatergic receptor sub-types appear to have actually been gradually *down-regulated* in chronic A + D cultures

Table 4
Spontaneous firing patterns as assayed in control *recording* medium (DMEM) following 3-weeks of culture in the presence of selective amino acid receptor blockers (BICUC and APV + DNQX groups)

	Control (13)	Bicuculline (9)	APV + DNQX (23)
MFR (sp/s)^	1.15–**1.47**–2.52	2.57–**3.77**–4.56*	0.44–**1.16**–3.56*#
Modal ISI (ms)^	5–**8**–27	3–**5**–9#	6–**20**–125**
Burst ratio^	0.67–**0.86**–0.93	0.94–**0.98**–0.98*	0.57–**0.77**–0.95##
CV 60 s (%)^	103–**109**–138	53–**67**–107*	47–**69**–99**
1000 ms criterion			
Duration (s)	1.27–**1.56**–3.19	0.48–**1.41**–1.90	0.33–**1.59**–4.70*
Intensity (sp/s)^	7.10–**10.5**–17.5	23.9–**34.6**–52.7**#	6.08–**16.9**–25.3*
Period (s/c)^	14.5–**15.2**–19.5	6.60–**7.50**–14.1*#	8.64–**11.9**–16.0*
100 ms criterion			
Duration (ms)^	57–**64**–127	129–**224**–266**##	61–**96**–136
Intensity (sp/s)	58.1–**65.7**–81.0	47.5–**74.3**–105	35.2–**51.7**–90.5*
Period (ms/c)	473–**532**–581	376–**478**–540	431–**505**–575
10 ms criterion			
Duration (ms)	9–**12**–13	12–**13**–20	9–**11**–14
Intensity (sp/s)	187–**225**–261	217–**230**–244	195–**248**–298
Period (ms/c)^	39–**46**–53	45–**46**–49	30–**32**––48**#

Abbreviations as in Table 1.
^Overall *treatment* effect (*$p < 0.05$).
**$p < .01$ vis à vis the control group.
#$p < 0.05$, ##$p < 0.01$ between the BICUC and APV + DNQX groups.

(Corner, 2008) in what may be considered 'forward reference' to the possibility of normal environmental conditions eventually being restored.

3.2. Chronic total glutamate receptor blockade

Hyperexcitability can be induced in isolated organotypic neocortex explants by chronically suppressing all synaptic transmission by means of a high extracellular magnesium concentration (see Baker et al., 1995; Corner, 1994). Blockade of AMPA along with NMDA receptors, too, causes a well-nigh total cessation of spiking, followed upon return to control medium by exaggerated spontaneous burst discharges (Bacci et al., 2001; Corner et al., 2002; Van den Pol et al., 1996). In co-cultures, however, the greatly enhanced dendritic arborizations (Baker and van Pelt, 1997) could be indicative of trophic factors, mediated by cortico-cortical projections, which render the growth of the developing networks less vulnerable to functional disturbances. In that event, experimental suppression of SAPs might produce little in the way of maturational deficits, thereby making positive *in vitro* results irrelevant for the intact brain. It is imperative, therefore, to confirm the ontogenetic role of spontaneous neuronal discharges in increasingly realistic 'model' systems before taking too seriously an extrapolation to the organism as a whole. It is this consideration which motivated our unsuccessful attempts to suppress SAP activity in cortical co-cultures by means of pharmacological blockade of excitatory synaptic receptors (see Section 2 and Corner et al., 2005). It was of great interest, on the other hand, to see the extent to which homeostatic restoration of spontaneous activity under such conditions would protect the developing network from becoming hyperexcitable (Corner et al., 2006).

Total glutamate receptor blockade (the 'LAD' group) led, already after 1-week *in vitro*, to a broad spectrum of deviations from control firing patterns when both groups were compared in normal growth medium (Table 6; Fig. 4 and see Corner et al., 2006). Despite a similar range of mean firing rates in the two groups, abnormalities included relatively long interspike intervals, lowered burst ratios, and shorter and/or less intense burst firing on all time-scales. It is striking that, from micro- to maxi-bursts (see Tables 6 and 9), chronically deprived cultures were characterized by exceptionally large variability from one specimen to the next: some explants were much less active than any of the controls, while others were extremely hyperactive; some showed relatively

Table 5
Average 'minute-order' fluctuations, recorded in control medium following chronic treatment with selective amino acid receptor blocking agents, as estimated by using a 10 s interspike interval criterion for inclusion of action potentials in the active phase of cycles on this time-scale ('*maxi-bursts*')

	Group				
	Control	BICUC	APV	DNQX	APV + DNQX
2 weeks in vitro	*n* = (19)	*n* = (15)	*n* = (21)	*n* = (18)	*n* = (12)
Duration (s)^	3.60–**28.4**–33.0	18.4–**102**–142*	18.0–**23.5**–29.8#	21.7–**42.1**–69.5**′	32.9–**36.4**–51.8**′
Period (s/c)^	44.4–**73.1**–93.2	68.3–**114**–182*	45.0–**71.1**–136#	50.5–**75.3**–103#+	92.6–**139**–172**′
3 weeks in vitro	*n* = (13)	*n* = (9)	–	–	*n* = (23)
Duration (s)^	17.8–**28.2**–68.9	28.3–**51.1**–116	–	–	33.1–**68.9**–8.0*
Period (s/c)	64.0–**93.7**–99.9	65.1–**69.5**–183	–	–	59.9–**84.3**–150

Abbreviations as in Table 1. Values are given as the *median* and the *50 percentile* range for the number of cultures given in parentheses.
^Overall treatment effect ($p < 0.05$).
*$p < 0.05$, **$p < 0.01$ for differences from the control group.
#$p < 0.05$ vis-à-vis the bicuculline group.
+$p < 0.05$ between the DNQX and the APV + DNQX groups.
′$p < 0.05$ between the APV and the DNQX or the APV + DNQX group.

Table 6
Effects of chronic blockade of kainate, APV and AMPA receptors in 1- and 3-week-old cultures ('LAD' groups) as assayed in normal growth medium (R16)

	1 week		3 weeks	
	Control (20)	LAD (31)	Control (16)	LAD (16)
MFR (sp/s)^	1.75–**2.52**–3.41	0.97–**2.87**–6.03**	2.50–**4.98**–8.48	4.82–**8.12**–9.18*
Modal ISI (ms)^	9–**10**–25	7–**25**–78**	2–**4**–7	5–**7**–9
Burst ratio^	0.88–**0.93**–0.96	0.64–**0.87**–0.95**	0.97–**0.98**–0.99	0.83–**0.93**–0.98*
CV-60 s (%)^	91–**102**–116	31–**53**–75**	52–**90**–131	69–**82**–90*
1000 ms criterion				
Duration (s)^	1.19–**1.76**–2.61	0.63–**1.58**–2.70	1.43–**3.94**–6.14	2.13–**3.98**–5.16
Intensity (sp/s)^	16.2–**24.6**–30.7	6.46–**15.9**–33.3**	29.9–**36.6**–44.9	12.3–**19.5**–37.9**
Period (s/c)^	9.81–**10.9**–20.4	4.71–**6.91**–12.9**	7.64––**18.7**–33.3	7.55–**8.80**–16.3*
100 ms criterion				
Duration (ms)	161–**204**–287	76–**160**–253	144–**190**–294	133–**239**–292
Intensity (sp/s)^	54.9–**60.3**–69.8	23.8–**37.9**–78.4**	73.7––**88.0**–103	55.4–**65.5**–92.6*
Period (ms/c)	443–**456**–528	375–**451**–517	398–**441**–459	462–**470**–544**
10 ms criterion				
Duration (ms)^	11–**13**–15	7–**9**–19**	11–**13**–21	14–**18**–20*
Intensity (sp/s)^	218–**225**–239	179–**221**–267	234–**259**–306	219–**232**–256*
Period (ms/c)^	41––**42** –45	41–**44**–46	42–**47**–51	41–**42**–43**

Abbreviations as in Table 1. See Table 7 for the 2-week-old data.
*$p < 0.05$, **$p < 0.01$ vis-à-vis the corresponding control group.
^$p < 0.05$ for age differences among the control groups.

short bursts of activity, while others had abnormally long bursts; and some were unusually stereotyped from one burst to the next, while others were unpredictable in the extreme. This is the picture one would expect if some of the explants were already well on their way to the predicted hyperexcitable state whereas others were only just beginning to compensate for the experimentally induced sluggish physiological activity. Such individual differences in the kinetics of homeostatic adaptation had indeed earlier been observed in experiments with isolated explants in which NMDA receptors were selectively blocked, and SBA then allowed to gradually recover on the basis of compensatory AMPA receptor up-regulation (Corner et al., 2002). Burst durations, on the other hand, are significantly less variable, and individual differences are less pronounced in chronic LAD-treated than in control co-cultures (data not shown), a pattern of behavior which is typical for highly immature cortical networks (Corner and Ramakers, 1992).

Large inter-individual differences also distinguish 2-week-old experimental cultures from the corresponding controls (Table 7), although not to the same extent as at 1-week *in vitro*. Significantly enhanced burst stereotypy (i.e., reduced coefficients of variation for burst durations: data not shown) is now present only on the mini-burst time-scale. The LAD-treated group shows no clearcut signs as yet of hyperexcitability or of exaggerated burst activity. Thus, despite prolonged midi-bursts which take up a greater proportion of the minute-order cycles, overall firing rates as well as micro- and mini-burst firing intensities are actually reduced (Fig. 2; Table 7). Similarly, although there is a significant shortening of burst periodicities on both the mini- and midi-burst time-scales (associated with a high incidence of bimodal distributions: Table 8), there is also a pronounced shift towards longer modal interspike intervals, along with an increase in the percentage of isolated action potentials between the spike clusters (see Table 7: *burst ratio*).

Table 7
Effects of chronic blockade of kainate, APV and AMPA receptors ('LAD' group) or of total suppression of action potentials (TTX group) as assayed in normal growth medium (R16) after 2-weeks of culture *in vitro*

	Control (25)	LAD (20)	TTX (15)	acu-BIC (12)
MFR (sp/s)^	3.08–**5.87**–7.21	1.28–**3.92**–6.98*	3.58–**5.08**–5.70	20.7–**21.8**–23.8**′′
Modal ISI (ms)^	2–**3**–6	8–**25**–200**	1–**2**–2**	5–**6**–7*′′
Burst ratio^	0.97–**0.99**–0.99	0.63–**0.88**–0.97**	0.96–**0.98**–0.99	0.96–**0.97**–0.98
CV-60 s (%)^	50–**69**–80	35–**52**–82′′	47–**54**–65*	43–**53**–62
1000 ms criterion				
Duration (s)^	0.24–**1.78**–4.45	1.57–**3.50**–7.07**′′	0.29–**0.39**–0.75**	3.14–**4.64**–7.54**′′
Intensity (sp/s)	35.1–**51.8**–71.3	7.30–**11.0**–23.3**′′	33.1–**40.0**–47.4**	43.7–**49.9**–55.9
Period (s/c)^	5.72–**10.6**–26.1	8.92–**15.0**–25.7′′	2.81–**4.11**–5.87**	7.44–**9.15**–15.9
100 ms criterion				
Duration (ms)	157–**202**–290	86–**165**–28′′	55–**65**–99**	266–**284**–320**′′
Intensity (sp/s)^	79.4–**104**–116	31.1–**51.6**–69.7**	102–**110**–135*′′	95.3–**112**–152
Period (ms/c)^	414–**456**–529	477–**516**–594*′′	223–**261**–294**	440–**470**–492
10 ms criterion				
Duration (ms)^	13–**16**–20	9–**12**–14**	9–**11**–12**	18–**22**–24*′′
Intensity (sp/s)^	244–**273**–301	186–**205**–229**′′	373–**392**–425**	294–**326**–362**′′
Period (ms/c)	42–**45**–47	40–**41**–43*′′	46–**50**–57*	41–**43**–43*′′

Abbreviations as in Table 1. acu-BIC = acute bicuculline treatment of control cultures.
^$p < 0.05$ for age differences among LAD groups (cf. Table 4).
*$p < 0.05$, **$p < 0.01$ vis-à-vis the control group.
′$p < 0.05$, ′′$p < 0.01$ vis-à-vis the TTX group.

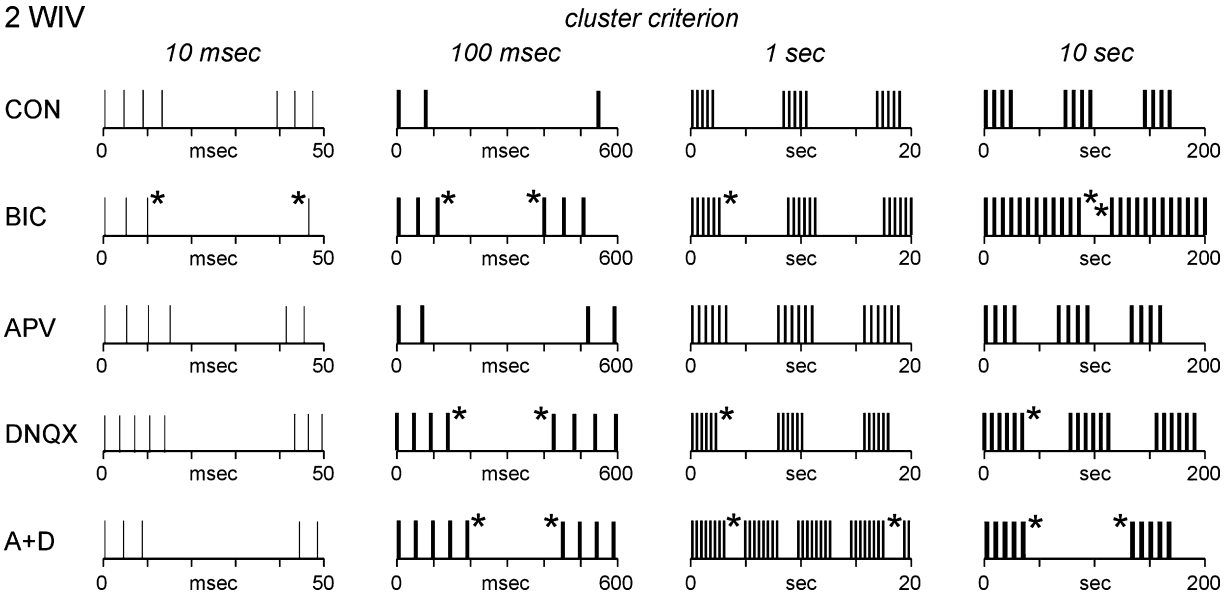

Fig. 2. Reconstructions for the representative spike trains, i.e., 'median' values calculated from the data in Table 3 for 'micro' (10 ms), 'mini' (100 ms), 'midi' (1 s) and 'maxi' (10 s) bursts – see Sections 1 and 5.2 for explanation of the spike cluster criteria – in cultures chronically treated with, respectively, bicucculine (BIC), APV, DNQX or both together (A + D) and then assayed in control medium at 2 weeks *in vitro*. Asterisks following a burst of action potentials signify statistically significant differences from control (CON) cultures with respect to duration and/or firing intensity; asterisks preceding a burst signify differences in periodicity (see corresponding table).

Burst distribution analysis (see Fig. 1) revealed several additional abnormalities in chronic LAD-treated cultures, all of them suggestive of an excitatory:inhibitory imbalance (Table 8). Thus, the majority of preparations show a tendency for midi-bursts (i.e., based on a 1000 ms interspike interval criterion) to recur at unusually short intervals, and many of them had a second peak at slightly longer inter-burst values (the relative height of the two peaks differed considerably from case to case: data not shown). Comparison of the burst histograms for mini- and micro-bursts with those of acutely disinhibited control cultures (Table 8: 'acu-BIC' group) is consistent with the hypothesis (see discussion in Corner et al., 2002) that lowered GABAergic synaptic activity is one of the major abnormalities caused by prolonged reduction of bioelectric activity during network maturation, brought about in the present experiments by ionotropic glutamatergic receptor blockade.

After 3-weeks of LAD treatment, the extreme inter-individual differences seen in younger LAD-treated cultures have disappeared, to be replaced by a consistently higher mean firing rate than in the controls (Table 6). On the other hand, both network and mini-burst durations are even more variable now than in control cultures (data not shown). Modal interspike interval values have fallen to the control values and burst ratios have become higher than they were at 1- and 2-weeks *in vitro*. Midi-bursts have become more frequent, while quiescent epochs during minute-order cycles are shorter than in control cultures (Tables 6 and 9). The incomplete SAP recovery in these cultures (see Fig. 4; and Corner et al., 2005) is evidently insufficient to prevent the gradual onset of hyperexcitability and augmented spike clustering within the maturing neocortical network.

As mentioned above, addition of a muscarinic receptor blocker to the growth medium during early network formation ('LADA'

Table 8
Analysis of dominant periodicities (see Fig. 3) in 2-week-old occipital cortex co-cultures recorded either in control medium after chronic reduction of spontaneous spike discharges (LAD and TTX groups) or in the presence of acutely added bicuculline to control cultures (acu-BIC group)

	Control (n = 24)[a]	LAD (n = 20)	TTX (n = 15)	acu-BIC (n = 12)
Burst criterion				
1000 ms (s)	1.5–**4.0**–7.5 (13)	3.0–**3.5**–5.5 (9)	2.0–**3.5**–5.5 (15)**	2.0–**3.0**–3.5 (9)**′
	–	1.5–**2.0**–2.5 (13)**′′	–	
100 ms (ms)	300–**450**–750 (17)	400–**550**–750(15)*′′	–	550–**700**–850 (8)*′′
	–	–	300–**300**–350 (7)*	325–**400**–450 (10)*+′′
	–	175–**250**–350 (15)**	150–**200**–250 (15)**	200–**250**–275 (9)**
10 ms (ms)	40–**55**–75 (16)	45–**55**–80 (5)**	60–**95**–115 (10)**++	55–**65**–80 (3)*+′
	20–**25**–30 (23)	15–**25**–35 (15)	15–**35**–45 (12)**	20–**25**–30 (12)′′

Abbreviations as in Table 1. acu-BIC = acute bicuculline treatment of control cultures.
Results are presented as the median and 100% range of identified peaks in the distribution histograms (see Fig. 3 and Section 1), with the numbers in parentheses giving their incidence within each group. The statistical tests compare the number of preparations in each group showing a periodicity within the indicated range of values (only a single value per preparation was admitted in each of the designated ranges).
[a] None of the 24 cases had secondary peaks, and 7 of them lacked any clear-cut peak at all in this range.
*$p < 0.05$, **$p < 0.01$ vis-à-vis control cultures.
+$p < 0.05$, ++$p < 0.01$ vis-à-vis LAD-treated cultures.
′$p < 0.05$, ′′$p < 0.01$ vis-à-vis TTX-treated cultures.

Table 9
Average 'minute-order' fluctuations, recorded in control medium following chronic total amino acid receptor blockade for 2 weeks *in vitro* with (LAD-A) or without (LAD group) the addition of atropine, as estimated by using a 10 s interspike interval criterion for inclusion of action potentials in the active phase of cycles on this time-scale ('*maxi-bursts*')

	Group			
	Control	LAD	LAD-A	Control
1 week in vitro	*n* = (20)	*n* = (31)		
Duration (s)	12.0–**21.0**–34.3	7.33–**33.3**–57.7*	–	–
Period (s/c)	44.0–**52.3**–94.1	65.4–**86.5**–102*	–	–
2 weeks in vitro	*n* = (25)	*n* = (20)	*n* = (6)	*n* = (20)
Duration (s)	9.73–**12.7**–23.2	18.8–**24.6**–31.6**	24.2–**31.8**–43.9	20.1–**28.1**–61.9
Period (s/c)	45.1–**62.3**–81.2	52.8–**60.2**–72.7	45.0–**51.1**–60.0	45.1–**53.3**–78.5
3 weeks in vitro	*n* = (16)	*n* = (16)		
Duration (s)	17.2–**22.6**–30.3	21.9–**24.6**–31.1	–	–
Period (s/c)	50.0–**72.9**–87.7	38.7–**50.8**–63.8*	–	–

Abbreviations as in Table 1.
*$p < 0.05$, **$p < 0.01$ vis-à-vis the corresponding control group.

group) prevents the attainment of control levels of spontaneous neuronal activity (Table 10). Nevertheless, when assayed after 2-weeks *in vitro*, these cultures, too, appear to have been to a large extent protected against becoming hyperactive. Thus, upon transfer to control medium, LADA-treated explants show little of the overall hyperactivity that typically follows prolonged tetrodotoxin (TTX) exposure (see below, and Corner et al., 2002): on the contrary, mean firing rates are even lower, and network- and mini-burst firing less intense than in control cultures (Fig. 6; Table 10). In addition, much greater mini- as well as micro-burst stereotypy is observed in the LADA group: the CV for burst durations falls consistently between 0.5 and 1.5, as opposed to 1.4–3.0 in most of the control preparations ($p < 0.01$ for both classes of bursts; data not shown).

The LADA group nevertheless resembles TTX-treated cultures (see below) with respect to (i) the relative shortness of spike bursts on all time-scales except for minute-order cyclicity ('maxi-bursts': see Table 9), and (ii) a higher incidence of midi- and micro-bursts than in the controls (Fig. 6; Table 10). In all of the above-mentioned respects, 'LADA' cultures deviate more strongly from control values than do LAD-treated explants, suggesting that the presence of enhanced cholinergic synaptic drive in the latter group contributes, despite the total absence of glutamatergic transmission, to the normalization of some aspects of physiological maturation. It is tempting to postulate that the tonic background firing induced by atropine in this experimental group (see Corner et al., 2005) contributes to the reduction in bursting.

3.3. Chronic suppression of action potential generation

Complete suppression of spontaneous spiking for several weeks, using the sodium channel blocker TTX, induces pronounced epileptiform burst discharges in dissociated neocortical cell cultures as soon as the toxin is washed out (Ramakers et al., 1990). A similar effect is observed in chronically TTX-treated organotypic co-cultures: intensified micro-bursts repeat themselves two or three times in succession so as to form mini-bursts lasting less than 100 ms each, as opposed to ca. 200 ms in the control group (Fig. 5; Table 7). These abbreviated mini-bursts occur in unusually rapid succession so as to form midi-bursts which,

Table 10
Spontaneous firing patterns in co-cultured neocortex explants at 2 weeks *in vitro*, recorded in a growth medium containing a tripartite 'cocktail' of glutamate receptor blockers supplemented by atropine ('LAD-A' group), followed by assay in control medium

	Recorded in			
	Growth medium		Control medium	
	Control (20)	LAD-A (26)	Control (20)	LAD-A (14)
MFR	2.67–**4.54**–5.62	0.98–**2.10**–3.89**	2.67–**5.44**–11.1	1.61–**3.01**–3.39**
Mode	4–**5**–7	8–**300**–8503	3–**5**–6	4–**5**–13
Ratio	0.88–0.**93**–0.97	0.39–0.**53**–0.83**	0.88–0.**93**–0.96	0.76–0.**87**–0.92*
CV60	79–**96**–159	41–**62**–92**	57–**103**–159	39–**48**–59**
1000 ms criterion				
Duration	1.71–**2.12**–2.40	1.04–**1.70**–3.82	1.71–**2.31**–2.96	0.83–**1.28**–1.77*
Intensity	22.4–**29.4**–48.0	3.09–**5.11**–11.8	19.9–**36.4**–48.3	12.0–**12.5**–18.1**
Period	12.5–**14.2**–24.2	7.34–**9.07**–17.0**	10.1–**17.9**–24.2	5.00–**6.74**–9.21**
100 ms criterion				
Duration	200–**281**–323	59–**94**–147**	197–**247**–300	63–**70**–78**
Intensity	62.5–**94.3**–114	29.3–**38.9**–62.4**	81.5–**105**–203	63.5–**85.7**–103*
Period	451–**481**–520	448–**513**–552	451–**471**–497	391–**457**––507
10 ms criterion				
Duration	14–**17**–23	5–**9**–12**	14–**16**–24	8–**11**–13**
Intensity	272–**296**–315	256–**262**–283*	333–**357**–375	273–**375**–385
Period	42–**44**–45	41–**43**–46	42–**44**–45	32–**38**–40**

Abbreviations as in Table 1.
*$p < 0.05$, **$p < 0.01$ for the LAD-A vs. the corresponding control group.

again, are abnormally short (ca. 400 ms vs. almost 2 s in the controls). These midi-bursts recur every 3–4 s (also see Table 8: 1000 ms criterion) in long trains which are interrupted only briefly every 1–2 min throughout recording sessions lasting up to 20 min. As expected, network- and mini-burst durations are significantly less variable in the TTX group, with coefficients of variation between 0.5 and 1.0 in most cases (control values often exceed 1.5; data not shown).

In most of the control explants, midi-bursts occur about as frequently as in TTX-treated explants (Table 7: 1000 ms criterion) but, in striking contrast to the latter, the burst trains tend to be interrupted every 10–15 s by long quiescent episodes (Table 9: controls at 2 wiv). The two groups differ also with respect to the much more rapid periodicity of mini-burst occurrence in TTX-treated cultures (Table 7: 100 ms criterion). In addition, a high proportion of their interspike interval histograms show a prominent secondary peak (see Corner and Ramakers, 1992), something seldom or never seen in controls. Although bimodal distributions in the micro-burst periodicity range are the rule for both groups (see Table 8: 10 ms criterion), the intervals between successive micro-bursts tend to be considerably longer in the TTX group, possibly in consequence of the intensified intra-burst firing following chronic TTX treatment (Fig. 5; Table 7). On the basis of these results, the possibility can be excluded that trophic effects on dendritic outgrowth (see Baker and van Pelt, 1997) render co-cultured explants less vulnerable than isolates to functional deprivation during cortical network formation.

Abnormal firing in chronically TTX-treated explants differs greatly from that induced by acute GABAergic disinhibition in untreated 2-week-old preparations, where abnormally long bursts of activity are seen on micro-, mini-, and midi-burst time-scales (Fig. 5; Table 7). Since the mean intensity of spiking throughout these long-lasting network bursts equals that during control bursts, while trains of network bursts occupy a much higher proportion of time (see Fig. 5: 10 s criterion), the overall firing rate is augmented almost fourfold in acutely disinhibited control cultures (Table 7: 'acu-BIC' group). Micro-burst firing intensities are also extremely pronounced in this group (Table 7: 10 ms criterion). In contrast to the TTX group, burst durations on all time-scales are significantly more variable in the acu-BIC group than in the controls (i.e., greater coefficients of variation: data not shown). The compensatory response of co-cultured cortical explants to chronic suppression of spontaneous spike activity thus differs in so many respects from the acute effects of GABAergic disinhibition that multiple mechanisms are undoubtedly involved (see Corner et al., 2002; Spitzer et al., 2005) over and above the originally postulated weakening of inhibitory synaptic activity (Corner and Ramakers, 1992; Ramakers et al., 1990, 1994). On the other hand, the similarities between these two groups with respect to the temporal organization of spontaneous burst discharges (see Table 8) support the hypothesis that partial reduction of inhibitory drive is an important contributory factor in TTX-induced hyperexcitability.

3.4. Chronic GABAergic synaptic blockade

Upon transfer to control medium, picrotoxin (PTX)-induced epileptiform discharges in dissociated neocortical cell cultures quickly become replaced by relatively 'tonic' spike trains, in which the incidence of stereotyped bursts is well below that observed in untreated control cultures (Ramakers et al., 1991). Thus, the homeostatic mechanisms leading to hyperexcitability in developing neuronal networks when SAP discharges are chronically suppressed (see above) can operate in the opposite direction as well. Since mean firing rates throughout the period of PTX-treatment are comparable to those in control cultures, it is the intensification of episodic rather than overall SAP activity which must be held responsible for the reduction in spontaneous bursting in favor of tonic firing, as compared with untreated cultures, after rescue from PTX exposure (also see Corner and Ramakers, 1992).

Isolated organotypic explants have not been studied in this respect, but a degree of homeostatic tempering of burst activity also takes place in co-cultured cortical explants. Thus, upon transfer to control medium after 2-weeks of exposure to bicuculline (BIC), an increase in low-level spiking is observed in between the bursts, along with a general shift of modal interspike intervals towards higher values (Table 3). Although overall mean firing rates in the treated cultures are virtually identical to control values, the temporal patterning of spontaneous activity differs markedly between the two groups: minute-to-minute fluctuations of the mean firing level are more pronounced in the BIC-group (see Table 3: CV-60 s) despite the tendency for there to be only short pauses between long trains of network bursts (Table 5). Especially striking is the greatly reduced inter-individual variance in most parameters (Table 3) so that, as was also true for dissociated cell cultures (Ramakers et al., 1991; Corner and Ramakers, 1992), chronically disinhibited explants appear to be accelerated in certain aspects of their functional maturation.

In certain other respects, however, chronic BIC-treated co-cultures display abnormalities in their firing patterns which constitute clearly deviant rather than merely accelerated development. Thus, although micro-bursts are somewhat shorter and less frequent than in the controls, average mini-burst durations become prolonged to well over 100 ms and follow each other at considerably shorter intervals (Table 3; Fig. 2). The high incidence of abnormally long mini-bursts is characteristic of 3-week-old experimental cultures as well, with their mean duration now being well over 200 ms (as compared with the 60–70 ms still being measured in control cultures at this age: Table 4; Fig. 3). In addition, together with higher burst ratios and steadier minute-to-minute firing levels, the mean SAP discharge rates in 3-week-old preparations are more than twice as high as in the control group (Table 4). The tendency for spontaneous spike activity to induce long-term *depression* (LTD) of burst firing – i.e., a negative feedback mechanism – thus becomes gradually replaced by long-term *potentiation* (LTP). There seems to be a parallel here to the ontogenetic transition from LTD to LTP in response to certain patterns of low-frequency tetanic stimulation, possibly in consequence of alterations in NMDA receptor localization or composition (Corlew et al., 2007; Lanté et al., 2006; Mao et al., 2006; Milner et al., 2004).

4. Synopsis of recent neocortical plasticity experiments

Intrinsically generated spike trains have in recent years, using a variety of *in vitro* 'model' systems, been implicated in the functional development of the neocortex. Moreover, the excitatory synaptic mechanisms driving 'spontaneous' neuronal discharges have been found to be capable of homeostatically adapting to experimental reductions in firing levels by rapidly up-regulating their respective contributions to ongoing activity. One (purpose) of the present paper is to review the experimental evidence for each of these phenomena, and to supplement it with previously unpublished data (see Section 3, which is summarized below).

[I] Spontaneously generated action potentials can be reversibly suppressed for long periods in dissociated cultures of developing rat occipital cortex neurons by exposing them chronically to TTX, a sodium channel blocker. In isolated organotypic cultures a similar effect can be achieved by combined blockade of AMPA and NMDA synaptic receptors ('A + D' group, treated chronically with APV and

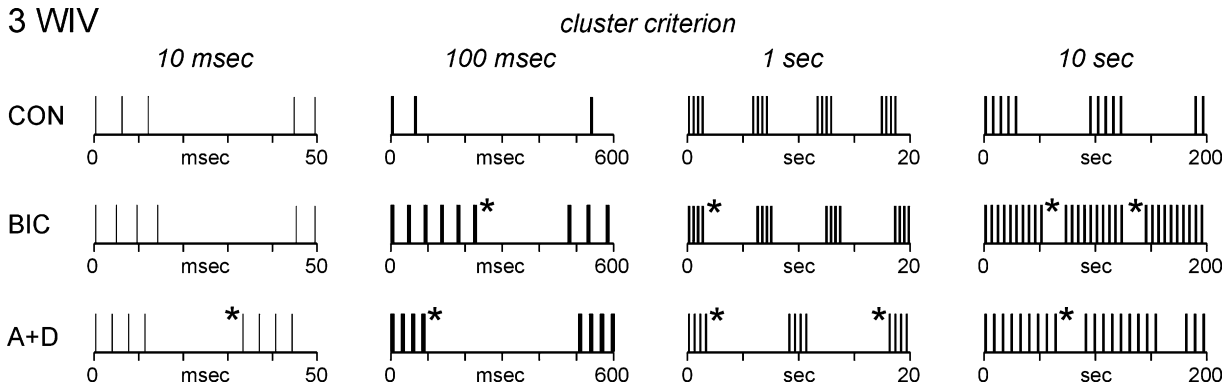

Fig. 3. As in Fig. 2, based on the data in Table 4 for chronically treated 3-week-old cultures.

DNQX). Selective blockade of NMDA receptors alone strongly suppresses spontaneous activity at first but, despite poor dendritic maturation in isolated cortical explants, AMPA receptors become able overnight to sustain SAP discharges at the control level.

If organotypic cortex explants are cultured pair-wise so as to permit contralateral cross-innervation, dendritic arborization and mean spontaneous firing rates begin to approximate *in vivo* values. The same is true for several basic aspects of immature brain function, such as the *tracé alternant* (Myers et al., 1997; Schwab et al., 2004) and 'cyclic alternating patterns' of slow-wave EEG activity (O'Brien et al., 1987; Terzano et al., 1988). In this improved *in vitro* 'model' system, chronic blockade of AMPA and/or NMDA receptors during network formation fails to prevent the appearance of SAPs, which are now maintained close to control levels via kainate receptor-mediated synaptic drive. Kainate receptors are not strictly co-localized with other glutamate sub-types and, during the induction of long-term potentiation at thalamo-cortical synapses *in vitro*, are capable of homeostatically regulating in the opposite direction as well (Kidd and Isaac, 1999).

Surprisingly, even with all three glutamate receptor sub-types chronically blocked ('LAD' group), SAP discharges in the co-cultured explants eventually attain a fairly normal pattern, albeit at a somewhat lower intensity of firing. Excitatory drive in LAD-treated cultures is found to have become largely cholinergic, with muscarinic but not nicotinic receptors now mediating the synaptically driven SAPs. Overnight exposure to the same 'cocktail' of excitatory amino acid blocking agents fails to mimic the effect of chronic treatment, with spontaneous firing recovering only slightly in that short a time. Even chronic blockade of muscarinic in addition to glutamate receptors fails to prevent the partial normalization of SAP activity in co-cultured cortex explants as they mature. Excitatory drive under these conditions becomes provided by an as yet unknown (non-nicotinic) source of synaptic input.

Although mean neuronal firing rates under several of the above-mentioned experimental conditions persist at close to control levels during the period of exposure (up to 3 wiv), burst discharges during exposure to the experimental growth media never acquire a fully normal pattern, with each of the chronic treatments having its own particular 'signature'. TTX is the only agent so far found to be capable, as in dissociated cell cultures, of permanently eliminating spontaneous spiking activity in co-cultured explants. Chronic GABAergic disinhibition (whether bicuculline or picrotoxin is used) has the effect in both types of preparation of enhancing stereotyped burst firing across a wide range of time-scales, while leaving mean firing rates largely unaffected over a period of several weeks.

[II] In dissociated cell cultures, chronic TTX-induced suppression of SAPs during early network formation leads, when assayed in control medium, to intensified spiking and exaggerated polyneuronal bursting which often has a highly regular character.

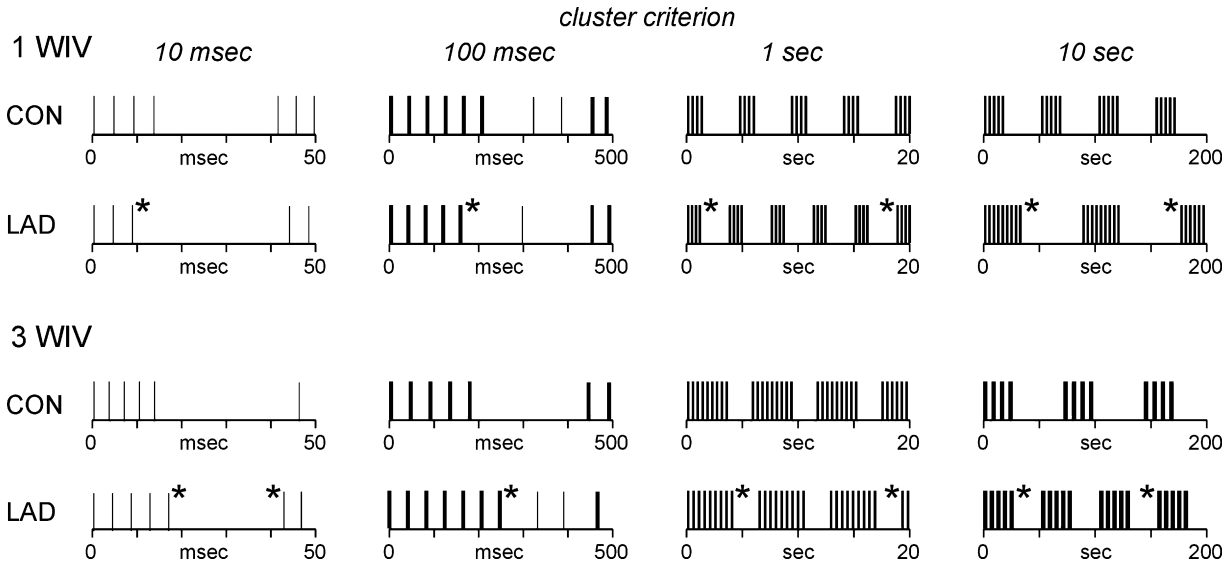

Fig. 4. As in Fig. 2, based on the data in Table 7 for cultures (LAD) subjected to chronic suppression of glutamate mediated spontaneous activity at 1 and 3 weeks *in vitro*.

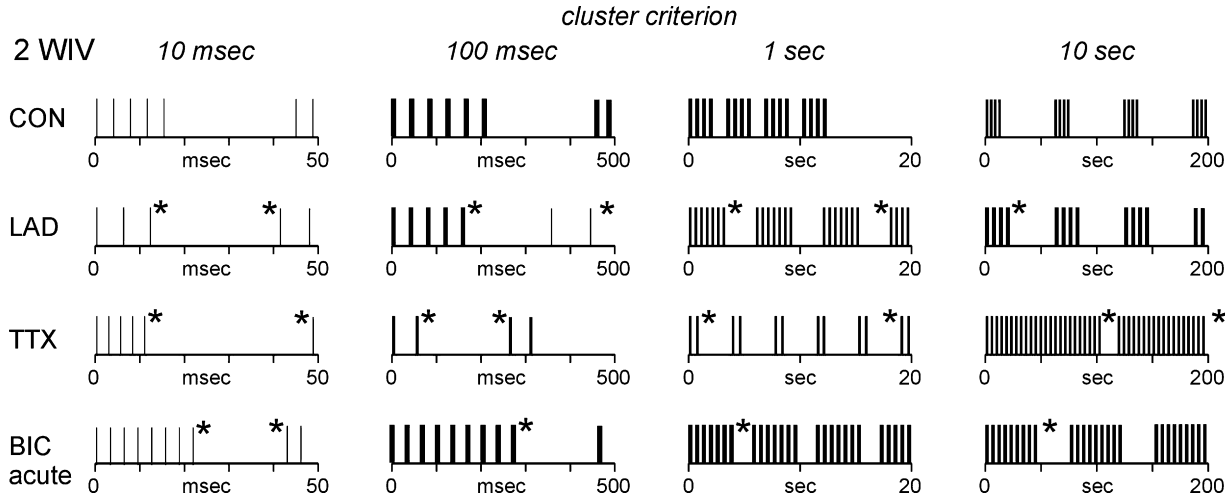

Fig. 5. As in Fig. 2, based on the data in Table 7, for cultures (LAD) subjected to chronic total suppression of glutamate mediated spontaneous activity at 2 weeks *in vitro*, as compared with chronic tetrodotoxin (TTX) and acute bicucculine (BIC) effects.

This hyperactivity can be attributed in large measure to deficient inhibitory synaptic maturation. Prolonged GABAergic disinhibition of such cultures has the opposite effect, viz., reduced burst stereotypy and lower firing rates when recorded in control medium. Elimination of SAP discharges in isolated organotypic neocortex explants by means of either elevated extracellular magnesium or combined AMPA + NMDA receptor blockade (A + D group) also leads to augmented spontaneous burst firing upon transfer to control medium. Interestingly, isolated explants become hyperactive also following several weeks of selective NMDA receptor blockade, despite normal levels of SAP activity throughout the treatment period.

In contrast, co-cultured explants (in which SAP firing rates are considerably higher than in isolated explants, both under control conditions and during chronic NMDA receptor blockade) show no signs of hyperexcitability upon transfer to control medium. Chronic AMPA receptor blockade, on the other hand, causes SAP bursting to be enhanced on several different time-scales when assayed in control medium several weeks later. This effect becomes more pronounced when AMPA and NMDA receptors have been simultaneously blocked. In neither case were SAP discharges less frequent or intense while in the experimental growth media than they were in control cultures, so that even high SAP firing levels can fail to completely normalize the development of network excitability when some of the receptor linked ion channels are prevented from participating.

Chronically LAD-treated explants (in which SAP levels during the treatment period are considerably less than in A + D treated explants) become hyperactive on all time-scales upon transfer to control medium. The greatest effect was seen after 3 weeks of such functional deprivation, with progressively larger deviations from control values appearing as the treatment period was extended from 1 to 3 weeks. Even then, the degree of abnormality is less – and qualitatively much different – than in cultures treated chronically with TTX (which already at 2 wiv display the same exaggerated burst stereotypy as reported for experiments involving dissociated neurons: see above). Chronic addition of the selective muscarinic receptor blocker, atropine, to the LAD growth cocktail causes a comparable reduction in SAP levels and to a firing pattern which, when assayed in control medium after 2-weeks *in vitro*, is too complex to be regarded as simply reflecting overall network hyperexcitability.

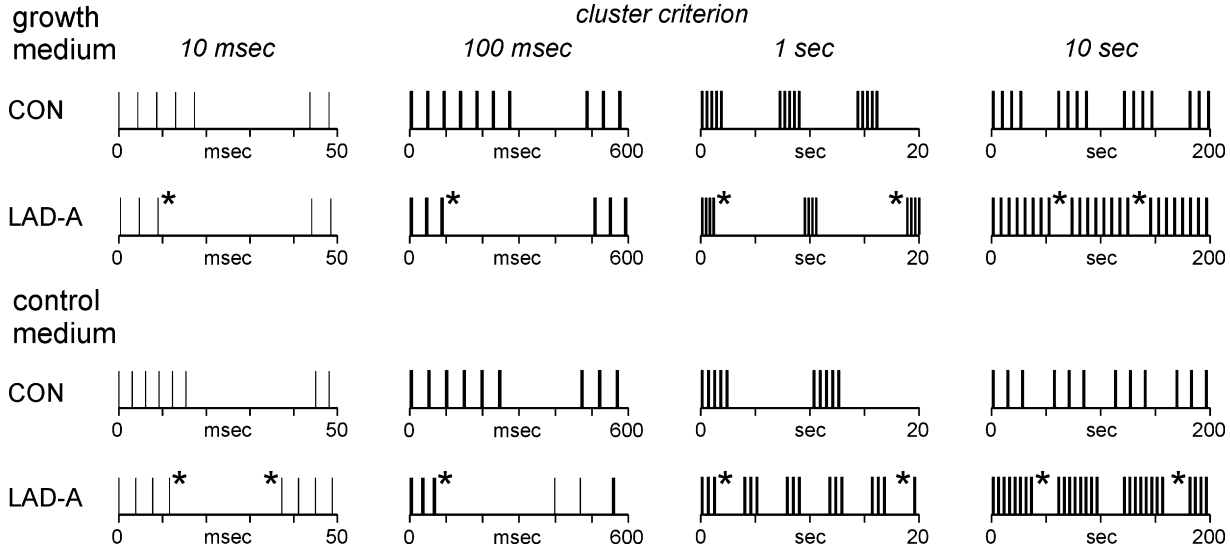

Fig. 6. As in Fig. 5, based on the data in Table 10, for cultures (LAD-A) subjected to chronic total suppression of glutamate, plus partial suppression of cholinergic (muscarinic) mediated spontaneous activity at 2 weeks *in vitro*, as assayed first in growth and then in control medium.

Chronic GABAergic disinhibition in developing dissociated neocortical cell cultures leads to a reduction in stereotyped bursting in comparison with untreated cultures recorded under the same (control) conditions after 2 wiv. Since this effect is the opposite of that induced by chronic TTX treatment, it appears from this result that functional homeostasis is a developmental phenomenon that can operate in both directions. In 2-week-old organotypic co-cultures, however, chronic disinhibition has several positive feedback effects on functional maturation – viz., the spike ratio declines, the interspike interval distribution shifts towards higher values, and minute-to-minute fluctuations in mean activity become more pronounced – along with parameters which behave as in dissociated cell cultures. For example, longer mini-bursts occur at shorter intervals than in the control group, thus producing intensified and prolonged midi-bursts. The latter take the form of long trains which are interrupted only briefly every few minutes, thus closely resembling chronic TTX-treated cultures in this respect.

After 3-weeks of continuous disinhibition, mini-bursts have become still longer and more intense, while long trains of intensified midi-bursts are interrupted only briefly about every 1–2 min. Mean firing rates and burst ratios are now well above control levels, while minute-order fluctuations (i.e., 'maxi-bursts') are greatly reduced. All in all, there has been a developmental shift from *depression* to *potentiation* of spontaneous burst activity in this *in vitro* 'model' system for neocortical maturation. The compensatory adaptation to prolonged epileptiform activity induced by the absence of inhibitory synaptic drive thus eventually becomes one of positive rather than of negative feedback.

[III] It may be concluded from the foregoing experiments that intrinsically generated neuronal discharges are crucial for normal electrophysiological development in a variety of neocortex 'model systems', but that even lowered levels of firing can afford partial protection against the epileptiform hyperexcitability which typically develops in the complete absence of SAP discharges. It is not surprising, therefore, to find that a wide variety of compensatory mechanisms exists to oppose any tendency for spontaneous discharges to become seriously reduced (e.g., Karmarker and Buonomano, 2006). Such homeostasis is far from perfect, however, which perhaps can account for the differential effects produced by each program of synaptic receptor blockade upon specific parameters in the complex temporal hierarchy of quasi-rhythmical spike bursts: see Section 3 for details. It is clear (also from comparison with the effects of acute GABAergic disinhibition) that, in co-cultures even more than in isolated organotypic explants, additional activity-dependent mechanisms must be operative besides the adjustment of inhibitory so as to balance excitatory synaptic drive.

Since co-cultured organotypic explants more closely approximate normal development *in vivo* than do neocortical explants grown in isolation, the intact immature brain may be presumed to be equally well buffered against reductions in excitatory synaptic drive, with all their potentially deleterious effects on neural network formation. This form of protective physiological homeostasis complements the relatively slow adaptation of blocked synaptic receptors to chronically altered firing levels, an adaptation which obviously can manifest itself only upon return to normal growth conditions. Furthermore, the homeostatic up-regulation of non-blocked excitatory receptors appears to weaken the inactivated ones, in compensatory 'forward reference' to the possibility of the latter becoming active at some time in the future (also see Corner, 2008). In contrast, epileptiform discharges induced by chronic reduction of inhibitory synaptic drive eventually come to have a positive rather than a negative feedback effect in the relatively complex co-culture system, thereby potentially aggravating pathogenic growth processes.

5. Empirical context and future research perspectives

5.1. Selection of suitable preparations ('model systems')

In his 'Introduction to Experimental Medicine', Claude Bernard argued for the use of the simplest preparation that retains the essential features for which one is seeking an explanation, i.e., what nowadays would be called a 'model system'. The burden of proof, however, remains for the investigator to establish that the properties of such models – especially their reaction to experimental manipulations – are in fact those that the system would have were it to be studied in an intact, fully mature or highly evolved organism (e.g., Corner, 1994; De Simoni et al., 2003; Goda and Davis, 2003; Gutiérrez and Heinemann, 1999). Intrinsically generated bioelectric activity within the central nervous system of developing vertebrates, in particular, is an especially striking phenomenon which continues to be manifested even when small fragments from a wide variety of regions are cultivated outside the body (exhaustively covered by Corner et al., in 2002, and since then partially reviewed by several other authors, including Ben-Ari, 2006a; Moody and Bosma, 2005; Spitzer, 2006).

The complex poly-rhythmicity of such spontaneous discharges is preserved to a varying degree in different kinds of preparation, with the best approximation so far to *in situ* neocortical firing patterns being obtained when two organotypic slices of rodent neocortex are co-cultured side-by-side in such a way as to enable them to cross-innervate one another (Baker et al., 2006). Such cultures typically display a complex pattern of recurring trains of low frequency field potentials (~1–6 Hz: 'delta/theta': Steriade et al., 1990) which are accompanied by phase-locked bursts of polyneuronal spike discharges (Corner et al., 2005, 2006, and the present report). Short trains (<2 s) of delta bursts have also been reported for organotypic prefrontal cortex fragments when co-cultured with basal forebrain and/or midbrain tegmental tissue (Seamans et al., 2003), while 'delta wave' episodes of fluctuating membrane activation have been observed during 'Up-states', lasting up to 2 s, in basal forebrain/cerebral cortex co-cultures (Kerr and Plenz, 2004). In contrast, as is also the case in small slabs of cortex isolated *in situ* (Timofeev et al., 2000a), isolated organotypic explants (regardless of size; Baker et al., 2006) and reaggregated neocortical cell cultures (Corner and Ramakers, 1992) typically display only intermittent single spike bursts, lasting well under 1 s, and separated by several seconds or more of inactivity. At the cellular level, on the other hand, even these simplified cortical networks preserve a wide range of basic functional as well as structural properties (reviewed in Corner et al., 2002; see further: Bettencourt et al., 2007; Colin-Le Brun et al., 2004; De Simoni et al., 2003; De Simoni and Edwards, 2006; Fellous et al., 2003; Holopainen and Lauren, 2003; Khazipov and Luhmann, 2006; Shu et al., 2007).

By cutting in the sagittal rather than the transverse plane, a considerable augmentation in explant size can be obtained ('mega-cultures': Baker et al., 2006), which has the additional advantage of enabling simultaneous recordings to be made from cortical areas as widely separated as occipital and prefrontal (see Baker et al., 2006). Whereas unilateral mega-slices show little or no improvement over isolated occipital cortex explants, co-cultured mega-explants show a further enhancement of SBA over that seen in co-cultures of occipital cortex only. Higher mean firing rates and enhanced minute-to-minute fluctuations are accompanied by increased 'background' firing between spike bursts (i.e., lower burst ratios) and by a more than twofold increase, on average, in

the duration of 'midi' bursts (Baker et al., 2006). Each such burst comprises a train of mini'-burst discharges in the 'delta/theta' frequency range (~1–6 Hz), lasting several seconds and interrupted by comparable intervals of greatly reduced activity. Since this pattern closely resembles the 'tracé alternant' EEG pattern characteristic of brain activity in sleeping human neonates (Myers et al., 1997; O'Brien et al., 1987; also see Terzano et al., 1988) as well as the repetitive bursts of 'persistent activity' seen in lightly anaesthetized rats (Seamans et al., 2003), the co-cultured mega-slice preparation indeed appears to give the best approximation of *in vivo* neocortical spontaneous activity so far achieved in an *in vitro* 'model' system. A good approximation to the normal slow-wave sleep pattern (as recorded simultaneously in surrounding intact cortical tissue) can also be attained with neuronally isolated slabs *in vivo* if they are sufficiently large (Timofeev et al., 2000b). Computer simulations have shown that such patterns are indeed a "fundamental and inherent property of noisy neural ensembles with sufficiently strong synaptic connections" (Holcman and Tsodyks, 2006).

As predicted (see Corner, 1964a), most burst parameters in the mega-co-cultures (except for the minute-to-minute changes in firing level, c.q., maxi-bursts) are considerably more consistent from preparation to preparation than in co-cultures made with occipital or prefrontal cortex only (Baker et al., 2006). Small networks are apparently extremely sensitive to the subtle epigenetic factors which determine cellular differentiation and maturation (see Corner, 1994; Gottlieb, 1998, 2002; McConnell, 1995; Nieuwkoop et al., 1985; O'Leary et al., 1995; Rubenstein et al., 1998; Ruiz i Altaba, 1994), presumably due to a strongly probabilistic basis for neurite outgrowth and synaptogenesis in quasi-random or scale-free connected networks (Chiappalone et al., 2007; Corner, 1994; Grinstein and Linsker, 2005; Le Bé and Markram, 2006; Marom and Shahaf, 2002; Stepanyants et al., 2004; Tsodyks et al., 2000; Van den Pol et al., 1998; Van Ooyen et al., 1992). Due to the high degree of functional interconnectivity which characterizes neuronal networks, larger systems would tend to be relatively well buffered against heterogeneity of local differentiation tendencies. Activity-dependent processes in particular could be averaged out by the tendency in early ontogeny for widespread network activation to be tightly synchronized regardless of intrinsic local differences in firing propensities (Droge et al., 1986; Feinerman et al., 2007; Jimbo et al., 2000; Robinson et al., 1993; Volgushev et al., 2006; Yvon et al., 2005). Once again, a good approximation to aspects of *in situ* physiology can be achieved by using the organotypic co-culture preparation which, by reducing the required number of cases, has the additional virtue of facilitating statistical testing for age and treatment effects (Baker et al., 2006).

It is noteworthy that, as a group, micro-burst parameters show little or no difference between isolated and co-cultured explants, suggesting that robust pre-programmed timing mechanisms may exist for this high-frequency range (and see Basu and Liljenstrom, 2001). Especially interesting in this regard is the observation that *beta/gamma* ($\gtrsim$15 Hz) along with *delta/theta* (~1–6 Hz) waves appear to be the fundamental components of intrinsic neocortical activity: they are present, namely, in all states of vigilance from deep sleep to strong arousal (Bullock and Basar, 1988; Freund, 2003; Steriade, 2006), with slow-waves predominating whenever the cortex is functionally isolated from afferent input (Corner et al., 2002; Hasselmo, 1995; Steriade et al., 1990; Wagenaar et al., 2005). Here too, organotypic co-cultures show an excellent approximation to physiological activities in the intact cerebral cortex (also see Blackwell et al., 2003; Kerr and Plenz, 2004; Traub et al., 2005; Whittington and Traub, 2003). A still better *in vitro* model for neocortical ontogeny ought to be obtainable by morpho-physiologically integrating cholinergic and monoaminergic neurons into the culture (see Tsai et al., 2002), since both of these sources of extrinsic innervation contribute importantly to cortical maturation (Bachman et al., 1994; Jaumotte and Zigmond, 2005; Kuczewski et al., 2005; Luo et al., 2003; Origlia et al., 2006; Robertson et al., 1997). Not all such improvements would necessarily be detectable, however, during the sleep-like state which normally predominates *in vitro* (see Baker et al., 2006; Corner et al., 2002, 2005). Finally, it bears mentioning that, except in the 'beta/gamma' frequency range (i.e., micro-bursts), prefrontal cortical areas seem to be consistently less active than occipital areas, possibly simply as a consequence of an intrinsic lag in maturation (Guillery, 2005) but are otherwise comparable in all respects (Baker et al., 2006).

It would be extremely useful to have an isolated brain preparation that not only 'sleeps' but also is capable of displaying the 'desynchronized' firing patterns characteristic of the physiologically aroused neocortex (Jouvet, 1967; Lopes da Silva, 1991; Steriade et al., 1990). Preliminary experiments in our lab involving the presentation of extensive brainstem material to developing cortical organotypic co-cultures (also see Gustafson et al., 2006; Guthrie et al., 2005) have given some indications for a partially 'aroused' pattern consisting of relatively tonic firing. Thus, the median 'bursting index' (see Wagenaar et al., 2005) calculated over the most active 5 min segment of 15 min recordings proved to be only 0.63 in preparations with added brainstem (range: 0.24–0.84, $n = 5$) as compared with 0.80 (0.59–1.00) for an equal number of control explants ($U = 3.5$, $p < 0.05$). With such an approach, mechanisms underlying slow-wave sleep-dependent consolidation of learned material (e.g., Dan and Boyd, 2006; Hoffman and McNaughton, 2002; Steriade and Timofeev, 2003) or re-adjustment of overall cortical excitability and/or circuitry (Frank et al., 2001, 2006; Tononi and Cirelli, 2006) could be approached using a stimulus-related plasticity protocol (Eytan et al., 2004; Marom and Shahaf, 2002; Shahaf and Marom, 2002) during a physiologically 'aroused' state, followed by a period of sleep-like 'brain wave' activity (see Wagenaar et al., 2005, 2006a,b). A reversible induction of tonic (i.e., sustained, non-bursting) firing has been reported in cultures subjected to circulating cholinergic agonists (Tateno et al., 2005a,b) but, since this is more a 'model' for paradoxical sleep than for wakefulness, it is likely to be less than ideal for carrying out realistic learning or memory experiments: the inclusion of monoaminergic in addition to cholinergic transmitters or agonists would be required, namely, for approximating the physiological state of the waking brain (see Adrien and Lanfumey, 1986; Corner, 1990; Hasselmo, 1995; Jouvet, 1972, 1999).

Over and above the greater accessibility which it gives for carrying out experiments which currently are technically unfeasible in intact animals, *ex vivo* culturing will continue to be valuable even after the technical problems have been overcome. Especially for ontogenetic studies, it will continue to be of interest to explore the inherent self-organizing powers of different parts of the central nervous system – or even of the entire system – in isolation from hormonal and other extra-neural factors, even when such potentialities are not directly relevant to understanding strictly normal development. Long-term maintenance *in vitro* is no longer a problem (for review, see Wagenaar et al., 2006a) but the survival of large or three-dimensional structures is still quite problematic. An infinitely graded bridge between standard culture techniques and the whole animal could be provided, however, by the application of chorio-allantoic membrane grafting in avian embryos cultivated *ex ovo* (see Corner and Richter, 1973). In such a preparation, a two-dimensional surface provides maximum accessibility for morpho-physiological observations of recombined mammalian tissue fragments of unlimited size and complexity, which can

retain their characteristic spatial organization owing to rapid vascularization and consequent sustained blood circulation. Furthermore, by early surgical elimination of most of the host embryo, a self-sustaining closed nutrient pumping system can be obtained which could persist for at least several months without any need for refreshment (M.A. Corner, unpublished observations).

It must nevertheless be kept in mind that experimental studies of activity-dependent development in a 'model' system, no matter how sophisticated, necessarily entail the danger of drawing unwarranted conclusions, and thus must eventually be verified in the intact organism. On the one hand, *false negative* results could mean simply that one or another intervening variable is missing on which physiological (bioelectric) activity crucially depends for its effectiveness (e.g. Fields et al., 1991), whereas a *false positive* result from an ontogenetic deprivation experiment can be expected if a factor which is normally present, such as a hormone, is missing which under normal circumstances would make bioelectric activity redundant (e.g., Baker et al., 1982). Nevertheless, if judiciously applied, model systems can appreciably facilitate the search for putative causal mechanisms that would justify a tedious (and always potentially fruitless!) attempt at confirmation in the infinitely more complex living organ system or whole animal (Corner, 1994).

5.2. Methodology for single channel time-series analysis

Our original intuition (Corner and Ramakers, 1992) that the analysis of peaks – i.e., preferred (instantaneous) frequencies – in the interspike interval histogram represents a useful approach to characterizing the burst structure of spike trains was once again confirmed in the present experiments by the revelation of many significant age-related trends in neuronal firing patterns. Even more to the point, the vulnerability of spontaneous spike trains to pharmacologic interventions designed to assess their activity-dependence during neural network formation was able to be convincingly demonstrated. Not only did simple burst-analysis once again prove to be far superior to mean firing rates and many other parameters for revealing differences among age and treatment groups (e.g., Corner and Ramakers, 1992; Habets et al., 1987), it also made possible a visual reconstruction of the main features of statistically representative spike trains in the different groups (see Corner et al., 2002, 2005, 2006, and the present report: Sections 2 and 3).

Using four preferred separation points, suggested by individual interval histograms for defining inter-burst versus intra-burst intervals (for an especially good example, see Corner et al., 2006; also Madhavan et al., 2007; Turnbull et al., 2005; Wyart et al., 2005), these reconstructions revealed a number of striking aspects of spontaneous spike patterning involving periodicities ranging from fractions of a millisecond to several minutes (also see Chiappalone et al., 2006, 2007; Grzywacz and Sernagor, 2000; Ikegaya et al., 2004; Segev et al., 2002, 2004; Wagenaar et al., 2005, 2006a,b). Specifically, bursting on the order of 20–25 Hz and 2–3 Hz (on the average) were brought to light, along with the previously reported burst incidence rates on the order of ~0.1 and ~0.01 Hz (Corner et al., 2002). We propose, therefore, to define these preferred frequencies of spontaneous neocortical activity as a hierarchy of nested polyneuronal discharges of increasing duration: *micro*-, *mini*-, *midi*- and *maxi*-bursts, respectively, with corresponding repetition rates in (i) the *beta*/*gamma* (e.g., Bullock and Basar, 1988), (ii) the *delta*/*theta* (Steriade et al., 1990), (iii) the '*slow*' (Steriade, 1997, 2006), and (iv) the '*infra-slow*' (Aladjalova, 1957, 1964) frequency ranges. [The last mentioned of these have also been called 'minute-order rhythms' (Corner et al., 2002, 2005; Habets et al., 1987), 'super, c.q., big bursts' (Madhavan et al., 2007; Wagenaar et al., 2006a,b), or 'metastable state transitions' (Sasaki et al., 2007).]

Suspected oscillations of still lower frequency, from >5 min up to ~24 h, such as are known to exist the intact brain – designated as, respectively, '*tonic*/*phasic*' (Corner, 1990; Orem, 1996; Takahara et al., 2002; Wehrle et al., 2007), '*circahoral*' (Brodsky, 2006; Stupfel and Pavely, 1990), '*ultradian*' (Corner, 1977; Lavie and Kripke, 1981) and '*circadian*' (Dijk and von Schantz, 2005; Mirmiran et al., 1995, 2003) rhythms – can be observed intermittently in long-term neocortical registrations from *in vitro* neocortex tissue (unpublished observations; also Van Pelt et al., 2004a,b; Wagenaar et al., 2006a) but seem not yet to have been systematically investigated. Fluctuations on the order of several hours have been noted for spontaneous motility in amphibian tadpoles (see Corner, 1977), so that this intermediate periodicity range between an hour ('circahoralian': see Brodsky, 2006) and a day ('circadian') deserves closer investigation.

Despite its proven discriminative value in the recent series of developmental experiments, use of interspike interval histograms for defining what is and what is not a 'burst' (also see Chiappalone et al., 2007; Gourevitch and Eggermont, 2007; Turnbull et al., 2005), followed by calculating the *mean* values for selected parameters, places severe limitations on the resolution of burst frequencies in the spike train. To begin with, distinct peaks must be consistently present in order for this method to have any objective basis at all for selecting the burst recognition criteria. Since, in addition, the location of peaks is never identical for all cases in a given group, a certain error is unavoidably introduced if each preparation is analyzed on the basis of preferred values in the group as a whole. Although one hesitates proposing to circumvent this problem by comparing age or treatment groups in which the computed values are based on optimized burst criteria for each individual preparation, the distribution of such optima might themselves serve as useful discriminatory parameters. In any case, by averaging all of the burst values calculated using histogram-derived criteria, only a single value is generated per parameter (e.g., incidence, duration, spike count and firing intensity, along with their respective variances: Corner et al., 2002) for each preparation within each pre-defined burst frequency range.

In reality, however, closely spaced multiple peaks in the distributions of periodicities and other spike cluster parameters are quite common no matter what criterion is used for defining a 'burst' (see Fig. 1 for examples of typical burst distribution spectra as observed in the present study; also Chiappalone et al., 2007). In such cases, of course, the mean values obtained for burst frequencies in a given preparation will not correspond to any of the modal values. Even when a single peak is present, moreover, the often highly skewed distribution of values for all parameters implies that mean value calculations will overestimate the 'correct' values based on the preferred incidence (i.e., mode) of burst durations, counts, intervals and periodicities (see Fig. 1). A more accurate estimate of this last parameter, in particular, is desirable since it should prove useful in considering putative mechanisms which, on the basis of their distinctive kinetics, could account for specific frequency components in spontaneously occurring spike trains (for discussion see Corner et al., 2002; Mangan and Kapur, 2004; Spitzer, 2006).

Various procedures have been reported for defining single- or multiple-unit bursts on the basis of integrated spike envelopes which rise above a specified baseline level and have a pre-determined duration, spike count or firing rate (Droge et al., 1986; Keefer et al., 2001; Madhavan et al., 2007; Martinoia et al., 2005a; Van Pelt et al., 2004a,b, 2005). Such transformations can also simplify the application of cross-correlation and other analog

time-series techniques for quantitatively analyzing the complex interactions occurring within neuronal networks (Beggs and Plenz, 2004; Chiappalone et al., 2007; Grzywacz and Sernagor, 2000; Le Feber et al., 2007; Segev et al., 2002, 2004). Since spike-associated local field potentials themselves largely reflect supra-threshold membrane fluctuations (Bove et al., 1996; Elul, 1972; Grinvald et al., 2003; Kerr et al., 2005; Lopes da Silva, 1991; Purpura, 1959; Whittington et al., 2000), and since such potentials are readily measurable in organotypic explants (see Aptowicz et al., 2004; Baker et al., 1995, 2006; Bausch et al., 2006; Beggs and Plenz, 2003, 2004; Corner et al., 2002, 2005; Crain, 1976; Mohajerani and Cherubini, 2005), field potential recordings represent yet another potentially useful approach to the tricky question of burst detection and characterization. Indeed, by virtue of the inclusion of sub-threshold synaptic activities, field potential measurements might even provide a more sensitive indicator of underlying neuronal interactions – albeit at the cost of reduced resolution at the neuronal level – than is obtainable using action potentials only. Similar considerations apply, of course, to the many published multi-channel optical recordings of neuronally generated calcium currents (Bacci et al., 2001; Corlew et al., 2004; Grinvald and Hildesheim, 2004; Onimaru and Homma, 2007; Sasaki et al., 2006, 2007; Spitzer, 2002, 2006; Van den Pol et al., 1996; Voigt et al., 2005; Wyart et al., 2005; Yuste et al., 1995; Yvon et al., 2005).

5.3. Temporal organization of spontaneous action potentials

Outgrowing neurites in all or most parts of the central nervous system begin early in their development, even before making synaptic contact with other cells, to be capable of spontaneously conducting action potentials and releasing transmitter molecules (reviewed in Corner et al., 2002; also Boukhaddaoui et al., 2001; Pieraut et al., 2007; Spitzer, 2002). A transient network forms within cerebral cortical structures in which intrinsically active, largely but not exclusively GABAergic, synaptic activity and neuronal spiking drives widespread synchronized bioelectric activity and calcium currents (Adelsberger et al., 2005; Ben-Ari, 2002; Canepari et al., 2000; Corlew et al., 2004; Dammerman et al., 2000a; De Lima et al., 2007; Hanganu et al., 2001; Hannou et al., 2002; Khazipov et al., 2004; Le Van Quyen et al., 2006; Liu et al., 2002; Menendez de la Prida and Sanchez-Andres, 2000; Owens et al., 1996; Voigt et al., 2001, 2005). Subsequently, sporadic glutamatergically mediated action potentials (initially triggered predominantly via NMDA receptors: reviewed in Corner et al., 2002; also see Golomb et al., 2006 as well as Section 5.3 of the present monograph) begin to appear here and there, the incidence and frequency of which increases progressively with development (Ben-Ari et al., 2004; Canepari et al., 2000; Chiappalone et al., 2006, 2007; Corner et al., 2002; Gozlan and Ben-Ari, 2003; Khazipov et al., 2001; Picken-Bahrey and Moody, 2003; Voigt et al., 2005; Wagenaar et al., 2006a).

Experiments in which synaptic interactions are pharmacologically blocked suggest that putative 'pacemaker' cells for driving this activity are, at best, few and far between (Corner, 1994; and see Bacci et al., 2001; Opitz et al., 2002) so that the origin of intrinsic bioelectric activity in the developing cerebral cortex apparently lies mainly in the depolarizing action of spontaneously released neurotransmitters (Burgard and Hablitz, 1993; Demarque et al., 2002; Ge et al., 2006; Hestrin, 1992; Kim et al., 1995; Lin et al., 2002; Pieraut et al., 2007; Wyart et al., 2005; Young and Poo, 1983). As immature membrane potentials only gradually attain their mature values (Bures, 1957; Christophe et al., 2005; Johnson and Buonomani, 2007; Kaspar et al., 1994; Kim et al., 1995; Mares, 1964; McCormick and Prince, 1987; Nakanishi et al., 1999; Reboreda et al., 2007; Zhang, 2004), firing thresholds could easily be low enough at first for spikes to be initiated locally on a purely stochastic basis.

Since, almost from the start, acute pharmacological blockade of synaptic inhibition increases the incidence of spontaneous neuronal discharges as well as lowering the threshold for electrically evoking them, inhibitory interactions must also constitute part of the initial ongoing 'background' activity in organotypic cultures of central nervous tissue (Corner and Crain, 1969, 1972; Crain, 1976) and also in immature acute slices and in intact neocortex, respectively (Purpura et al., 1965; Wells et al., 2000). Bicuculline sensitive hyperpolarizing spontaneous membrane fluctuations indeed persist even during tetrodotoxin-induced spike blockade (Galvan et al., 1985; Kilb and Luhmann, 2001) as well as being present even before synaptic contacts are made (Gao and van den Pol, 2000; Munno and Syed, 2003) and in autaptically self-innervated GABAergic neocortical interneurons (Palizvan et al., 2004). Already at very early stages of maturation, therefore, distributed feed-forward inhibition must be a contributory factor regulating the excitability of the developing cerebral cortex (also see Johnson and Buonomani, 2007; Thompson et al., 2007; Wells et al., 2000). Overall network excitability is thus ontogenetically regulated by the opposing forces of, on the one hand, increasing excitatory interactions and, on the other hand, declining neuronal susceptibilities for spike initiation, with the latter being a function not only of the gradual enhancement of inhibitory synaptic activity (e.g., Crain, 1976; Luhmann and Prince, 1990b, 1991; Swanwick et al., 2006b; Wells et al., 2000) but also of the increasing hyperpolarization of neuronal membrane potentials. An additional contributory factor early on, in both archi- and neocortex, is the conversion of GABAergic synaptic drive from excitatory to inhibitory (Ben-Ari, 2002; Fiumelli and Woodin, 2007; Khazipov et al., 2004; Le Van Quyen et al., 2006; Mohajerani and Cherubini, 2005; Nakamura et al., 2007; Sun, 2007; Voigt et al., 2001).

As the density of excitatory interconnections increases, developing neural networks reach a point where a critical level of background activation triggers all-or-none bursts of reverberatory and feed-forward polyneuronal spike activity, accompanied by slow synaptic and field potentials (Beggs and Plenz, 2003, 2004; Bove et al., 1996; Corner et al., 2002; Crain, 1976; Johnson and Buonomani, 2007; Kano et al., 2005; Menendez de la Prida and Sanchez-Andres, 2000; Rutherford et al., 1997; Voigt et al., 2005), which propagate so as to recruit neurons at varying distances from the site of initiation (Ben-Ari, 2006b; Chiappalone et al., 2007; Eytan and Marom, 2006; Golomb and Amitai, 1997; Jimbo and Robinson, 2000; Madhavan et al., 2007; Maeda et al., 1998; Massimini et al., 2004; Onimaru and Homma, 2007; Pinato et al., 1999; Tateno et al., 2005b; Tsodyks et al., 2000; Van Pelt et al., 2005). This strong propensity towards repetitive spontaneous all-or-none burst discharges, characteristic for systems poised in a 'self-organized critical state' (Beggs and Plenz, 2003, 2004; Plenz and Thiagarajan, 2007; Teramae and Fukai, 2007), is in fact pre-programmed into the developing central nervous system already at the earliest neurula stages (Corner, 1964a,b; Corner and Crain, 1965). It thus represents, albeit with regionally specific, age dependent and inter-individual variations, a primordial functional activity pattern from which later appearing behavioral and neurophysiological manifestations are either derived or which they supplant (see Corner, 1977, 1994).

Thanks to spectacular advances in molecular biological techniques, renewed interest has arisen in recent years in mapping the regional distribution of developmental propensities within the presumptive central nervous system (Altmann and Brivanlou, 2001; Chitnis, 1999; Kobayashi et al., 2002; Lumsden and Krumlauf, 1996), with considerable attention being directed

towards the prospective forebrain area, including the cerebral cortex (Donohue and Rakic, 1999; Pallas, 2001; Rubenstein et al., 1998; Wilson and Houart, 2004). A program of parallel electrophysiological and cyto-morphological experiments (e.g., Corner, 1963, 1964a; Corner and Crain, 1965; Peng et al., 2007) as initially pluripotent proto-neural cells become progressively restricted in their capacities for molecular differentiation (Braun et al., 2003; Desai and McConnell, 2000; Gunhaga et al., 2003; Slack and Tannahill, 1992) would surely pay dividends in terms of a deeper understanding of the ontogenetic roots of brain function and behavior (Corner, 1990,1994), a subject which even now is passed over summarily in most neuro(bio)logical textbooks. This blindspot is especially true as regards the largely intrinsic biorhythmical basis of primordial neurophysiological activity as well as its persistence in later life as sleep cycles (discussed at length in Corner et al., 2002; also see Baker et al., 2006).

Both in dispersed neocortical cell cultures (e.g., Corner and Ramakers, 1992) and in single organotypic neocortical explants (Baker et al., 2006) – even when co-cultured with basal forebrain tissue (Blackwell et al., 2003) – but also in small neuronally isolated slabs *in situ* (Timofeev et al., 2000b) and in realistic computer simulated cortical networks (Bazhenov et al., 2002), the 'mini-bursts' usually last only a few hundred milliseconds at the most before a silent interval of several seconds or longer intervenes (Beggs and Plenz, 2003, 2004; Buckby et al., 2006; Buonomano, 2003; Chiappalone et al., 2006, 2007; Corner and Crain, 1972; Lauri et al., 2003; Madhavan et al., 2007; Opitz et al., 2002; Sasaki et al., 2007). In artificial cerebrospinal fluid, however, spontaneous 'midi-bursts' often last for 1–2 s or more (Compte et al., 2003; Johnson and Buonomani, 2007; Le Bé and Markram, 2006; Sanchez-Vives and McCormick, 2000). In contrast, isolated hippocampal neurons with exclusively glutamatergic autaptic synapses tend to re-excite themselves, depending on their age *in vitro*, either repetitively every 5 s or so in the form of midi-bursts lasting a few seconds (Takada et al., 2005) and phasically subdivided into mini-burst sequences (Boukhaddaoui et al., 2001), or else tonically for up to 10–20 s at a time with little indication of spike clustering (Wyart et al., 2005). Burst termination is characteristically caused by a prolonged, potassium channel-dependent, post-burst relative refractoriness whereby the threshold for evoking a new burst slowly declines with a time-course encompassing the range of spontaneous interburst intervals observed among different preparations, depending on their excitability and level of background firing (Corner and Crain, 1969,1972; also see Corner et al., 2002; Golomb and Amitai, 1997; Harris et al., 2002; Latham et al., 2000; O'Donovan and Rinzel, 1997; Wenner and O'Donovan, 2001).

Co-cultured neocortex explants, on the other hand, are characterized by short trains of mini-bursts at 400–500 ms intervals, on the average (Corner et al., 2005, 2006, and the present report: see Sections 2 and 3), with each train defining a 'midi-burst' of delta/theta oscillations (Lopes da Silva, 1991; Steriade et al., 1990; and see Section 5.2). This pattern is especially striking in 'mega'-co-cultures (Baker et al., 2006), where variable midi-bursts lasting several seconds alternate with silent intervals of comparable duration – i.e., the 'slow cortical rhythm' of Steriade (1997, 2006; also Picken-Bahrey and Moody, 2002) – in mimicry of the *tracé alternant* EEG pattern seen in sleeping neonatal infants (Myers et al., 1997; Schwab et al., 2004). In all types of preparation, periodic fluctuations of high and low spontaneous activity on the order of 1–3 min – 'maxi'-burst cycles, in other words, corresponding to the 'infra-slow oscillation' of Aladjalova (1957, 1964) – are commonly encountered (e.g., Corner et al., 2002, 2005; Le Bé and Markram, 2006; Voigt et al., 2005) while, at the other extreme, each mini-burst tends to be further subdivided into 'micro'-bursts at ~40–50 ms intervals on the average (Chiappalone et al., 2006; Corner et al., 2005; also see Yvon et al., 2005): i.e., beta/gamma oscillations: Lopes da Silva, 1991; Steriade et al., 1990; also see Section 5.2 of the present report.

Application of the burst distribution method of analysis to co-cultured *mega*-explants (see Baker et al., 2006) has revealed an unexpected richness of rhythmic components. Thus, whereas in occipital cortex co-cultures a single preferred periodicity was present somewhere within the 1–10 s/cycle range (also Chiappalone et al., 2007), and even then in only half of the cases (see Table 8), mega-(co)cultures consistently exhibit frequent episodes where, at both prefrontal and occipital recording sites, such 'midi'-spike bursts follow one another already at <2 s intervals (unpublished observations). Half of these 'mega' cultures, furthermore, show a second preferred periodicity (midi-bursts at 3–4 s intervals) while a few of them had a third peak in the ~6 s range as well. Bi- and tri-modal distributions are prevalent also in the 'mini'-burst periodicity range (i.e., 100–1000 ms), again in stark contrast to cultures of occipital cortex only (see Table 8). In the 10–100 ms range, on the other hand, bimodal distributions are relatively infrequent (unpublished observations), thus being indicative of an enhanced tendency in mega-cultures for 'micro-burst' components of the spike train to follow each other at regular intervals with few pauses in the burst train.

Although synaptic excitation is initially mediated largely by depolarizing GABAergic potentials (Corlew et al., 2004; De Lima et al., 2007; Opitz et al., 2002; Voigt et al., 2001), by the time that quantifiable spike trains appear in neocortical tissues cultured *in vitro*, ionotropic glutamatergic synaptic transmission is the only noticeable source of excitatory interactions (e.g., Canepari et al., 1997; Corner et al., 2002, 2005; Kim et al., 1995). Thus, a cocktail of AMPA and NMDA receptor blockers suffices to completely eliminate spontaneous spiking within minutes after application. NMDA receptors provide the lion's share of the cortical excitatory drive at first but their contribution gradually become overshadowed by AMPA receptor driven activity (Corner et al., 2002, 2005; Golomb and Amitai, 1997; Kumar et al., 2002; Luhmann and Prince, 1990a; Minlebaev et al., 2007; Partridge et al., 2000; Ramakers et al., 1993; Renger et al., 2001; Wang and Jensen, 1996). Selective blockade of kainate, muscarinic or nicotinic receptors has no apparent effect on spontaneous firing in untreated control cultures (Corner et al., 2005). Peptidergic neurons are scattered throughout organotypic neocortex slice cultures (Gibson et al., 2006; Goetz and Bolz, 1994) but their possible contribution to spontaneous bioelectric activity has not yet been investigated. Along with cholinergic neurons, they would be candidates for the low-level 'autonomous' spiking reported to persist in neocortical slices under conditions of glutamate receptor blockade (Mao et al., 2001).

The degree and intensity of neuronal involvement in synchronized bursting activity is controlled by the extent to which inhibitory neurons effectively participate in the response (Bosman et al., 2005; Fiumelli and Woodin, 2007; Heinen et al., 2004; Kudela et al., 2003; Minlebaev et al., 2007; Thompson et al., 2007). In largely excitatory networks, such as at the onset of spontaneous bursting or in the presence of inhibitory receptor blockers (reviewed in Corner et al., 2002), individual bursts terminate by themselves after a few hundred milliseconds at the most. In cultured neocortical networks, the stereotyped midi-bursts become longer, less intense and more variable as inhibition gradually becomes stronger, as demonstrated by their rapid reversion to stereotyped shorter and more intense bursts immediately upon GABAergic disinhibition (Corner and Ramakers, 1992; Ramakers et al., 1990). At a still later stage, in contrast, disinhibition causes spontaneous cortical bursts to become

considerably prolonged (Chiappalone et al., 2007; Corner et al., 2002), suggesting that inhibitory synaptic activity eventually comes to involve powerful feedback mechanisms (also Shu et al., 2003b; Volgushev et al., 2006). Feed-forward inhibition, in contrast, appears to become weaker with development (also Johnson and Buonomani, 2007), judging by the abrupt appearance of each network burst without any noticeable preceding buildup of background spiking (Chiappalone et al., 2006; van Pelt, 2004a,b). This is a likely expression at the network level of the reduced efficacy of inhibitory synaptic potentials as a result of developmentally enhanced membrane kinetics (Bosman et al., 2005; Heinen et al., 2004).

Acute application of the muscarinic receptor blocker, atropine, leads to intensified network bursts superimposed on a continuous background of low-level spike activity (Corner et al., 2005). Intrinsic cholinergic neurons are scattered throughout the neocortex (Bandyopadhyay et al., 2006; Houser et al., 1985; Schambra et al., 1989) and appear to exert a mild tonic inhibitory action (Corner et al., 2005). In order to verify the likelihood of cholinergic mechanisms being active also in mega-cortex explants, recordings of spontaneous activity were made in the presence of atropine (Baker et al., 2006). The results confirmed the existence of an inhibitory cholinergic component also in these preparations: greatly enhanced burst activity appeared almost immediately against a background of persistent background firing which had hitherto been absent. It is noteworthy that, in most cases, a clearcut '(midi-)burst-pause' pattern consisting of short episodes of intensified spiking every few seconds could still be revealed by computer analysis. Equally striking was the persistence of detectable delta/theta and beta/gamma frequency patterning – i.e., sequences of 'mini'- and 'micro'-bursts, respectively – within the spike trains despite the potentially confounding effect of ongoing 'tonic' firing (unpublished observations). Such robustness of specific oscillatory mechanisms, both slow and fast, constitutes still further evidence of an ontogenetically as well as phylogenetically (see Bullock and Basar, 1988) conserved aspect of neocortical network organization (Buzsaki and Chroback, 1995; Freund, 2003).

5.4. Spatial distribution of spontaneous neuronal discharges

Some of the parameters so far reported in explant cultures, such as the incidence of 'midi'-bursts and the periodicity of minute-to-minute fluctuations in mean firing rate (i.e., 'maxi'- burst cycles: see Section 5.2), are distinctive properties of a given preparation by virtue of the usually high degree of synchronous firing during each such burst or slow oscillation (Corner et al., 2002, 2005). Little would be gained with respect to such parameters by sampling more than a single point in a given preparation. Much of the inter-individual variance in other variables measured, however, is an expression of regional differences within each explant, as is readily ascertainable by recording either sequentially (see Baker et al., 2006; Crain, 1976) or from several electrodes simultaneously (e.g., Beggs and Plenz, 2003, 2004; Bettencourt et al., 2007; Chao et al., 2007; Droge et al., 1986; Giugliano et al., 2004; Jimbo and Robinson, 2000; Krause et al., 2006; Madhavan et al., 2007; Potter and DeMarse, 2001; Rolston et al., 2007; Wagenaar et al., 2006a,b). Thus, the mean duration, intensity and oscillatory character of a midi- or maxi-burst tends to be unpredictably site-specific, even in mega-co-cultures (Baker et al., 2006), thus giving each age and treatment group an irreducibly large variance with regard to those parameters (Echevarria and Albus, 2000; Pinato et al., 1999; Wagenaar et al., 2006a). That such intra-group variability has not precluded statistical discrimination among groups of cultures subjected to different levels and patterns of neuronal activity during the period of network formation (Corner et al., 2002, 2005, 2006) is in itself a testimony as to how strongly spontaneous firing is capable of influencing functional development in the cerebral cortex.

It would therefore be desirable in future investigations to be able to average out local regional variations and, so, to arrive at a value for each calculated variable which would give better consistency among preparations of a given age or treatment group. Thus, following the example of several groups before us (for review, see Van Pelt et al., 2005), dissociated fetal rat neocortical cells were cultured on 60-electrode planar arrays, and their spontaneous activity followed continuously over a period of 1–2 months (Van Pelt et al., 2004a,b). Plots of the individual time-stamps for simultaneously recorded action potentials during successive bursts confirmed the high degree of neuronal variations in duration, intensity and discharge pattern recently documented by Wagenaar et al. (2006a; also see Harsch and Robinson, 2000). The patterning at each site, on the other hand, proved in these studies to be remarkably constant from hour to hour, with highly stable phase relationships existing among the participating neurons (also Beggs and Plenz, 2004; Ikegaya et al., 2004; Madhavan et al., 2007; Rolston et al., 2007; Wagenaar et al., 2006b). In addition, the integrated spike activity profile for a given network, averaged over all bursts during consecutive 4-h epochs, showed great consistency over many hours (and often even days) of continuous recording: Van Pelt et al., 2004a,b, 2005; also Wagenaar et al., 2006a,b; but see Johnson and Buonomani, 2007 for a different interpretation.

The integrated burst profiles for the total measured spike activity throughout such dispersed rodent neocortical cell cultures are similar at comparable stages of development, and show similar developmental trajectories (Van Pelt et al., 2004a,b, 2005). Thus, once initiated, network ('midi-') bursts at first show a rapid overall build-up of firing intensity to a peak which then decays more slowly, the burst as a whole lasting 1–2 s. During the third week *in vitro* the bursts evolve into a longer overall discharge (3–4 s), with a slower rise time to the peak firing frequency. In all five cases studied, progressive shortening during the fourth week resulted in a thereafter highly stable pattern of population bursts, which were shorter than 1 s and were characterized by extremely rapid rise and decay times (also see Chiappalone et al., 2004). This high degree of intra-group consistency using MEA technology means that fewer cases per age or treatment group will need to be statistically analyzed in many future investigations of activity-dependent development. [Preliminary experiments in our lab have indicated that organotypic neocortex (co-)cultures go through a similar developmental sequence during the second week *in vitro*: unpublished observations.]

That the above-mentioned maturational change may be dictated by the spontaneous activity itself is suggested by recent experiments in which repetitive tetanic stimulation was able, within hours, to cause a persistent transition to a prematurely foreshortened network burst profile (Van Pelt et al., 2005; also see Madhanva et al., 2007). It would therefore be of interest to see if this change fails to occur in cultures treated with, for example, tetrodotoxin so as to suppress spontaneous spiking during the normal transition to the mature pattern of compact bursts. In light of the fact that burst durations in mature occipital cortex cultures are greatly lengthened by the acute administration of GABAergic receptor blockers (Corner et al., 2002), inhibitory feedback is likely to become a major contributory factor to burst attenuation as development proceeds. Indeed, synaptic inhibition is known to be one of the prime activity-dependent mechanisms throughout the developing central nervous system (reviewed in Corner et al., 2002; further, Chattopadhyaya et al., 2004; De Lima et al., 2004,

2007; Issa, 2003; Kotak and Sanes, 2003; Liu et al., 2002; Palizvan et al., 2004; Rosato-Siri et al., 2002; Tao and Poo, 2005).

Time-plots of network-wide spike activity between successive bursts have revealed several additional age-specific patterns in cultured rodent neocortical networks (Van Pelt et al., 2004a,b, 2005). Thus, whereas in immature cultures the network bursts are typically initiated by a slow ramp-like build-up of firing at one or a few sites, this activity profile progressively gives way over the next few weeks to a durable pattern of (shortened) bursts with an abrupt onset every few seconds and with no noticeable after-discharges. Such changes in spontaneous firing suggest that feed-forward excitatory synaptic interactions within developing cortical networks become stronger during the same period that effective inhibitory feedback comes into play. Since the numerical density of spine synapses actually declines in this period (Van Huizen et al., 1985, 1987a), the remaining excitatory connections would appear to have become more efficient in evoking post-synaptic spiking, possibly in part as a result of lowered background levels of spontaneous inhibitory interactions (Anderson et al., 2004; Kilman et al., 2002; Rosato-Siri et al., 2002). Earlier studies with a variety of cultured CNS explants had already demonstrated that the threshold for triggering generalized bursts of neuronal spiking is indeed a function of ongoing inhibitory synaptic activity, and falls dramatically with age *in vitro* (Corner and Crain, 1969, 1972; also see Crain, 1976). At the same time, the virtual absence of interburst spike activity at later stages could be indicative of a concomitant developmental decline in the probability of excitatory transmitter release (Chavis and Westbrook, 2001; Takada et al., 2005; Wasling et al., 2004).

Multi-electrode recording techniques offer, of course, the additional advantage of quantifying each channel separately so that local interactions within a neural network can be studied (e.g., Chao et al., 2007; Li et al., 2007a,b; Van Pelt et al., 2004a,b, 2005). Such studies have revealed that bursts are initiated probabilistically from different locations in dispersed neuronal cell cultures, but that the incidence of such 'leading sites' can vary considerably (Droge et al., 1986; Jimbo et al., 2000; Menendez de la Prida and Sanchez-Andres, 2000; Robinson et al., 1993; Tsodyks et al., 2000; Volgushev et al., 2006; Yvon et al., 2005; also see Compte et al., 2003; Massimini et al., 2004). An especially intriguing finding is that, although the relative contribution of individual neurons to the overall network activity can vary enormously at different stages of development, their average phase relationships during burst discharges are often remarkably constant even over many weeks (Van Pelt et al., 2004a,b, 2005). Apparently, even in quasi-randomly reassembled reaggregates as well as in isolated 'organotypic' cortical networks, neurons begin very early on to form precise interconnections which, at least during spontaneous bursts of network-wide activity, form the basis for stable 'chains' of sequential activation (Bettencourt et al., 2007; Buonomano, 2003; Cossart et al., 2003; Ikegaya et al., 2004; Johnson and Buonomani, 2007; Kozloski et al., 2001; Le Feber et al., 2007; Luczak et al., 2007; MacLean et al., 2005; Madhavan et al., 2007; Segev et al., 2002, 2004; Wagenaar et al., 2006b – but cf. Kerr et al., 2005; McLelland and Paulsen, 2007; Mokeichev et al., 2007; Sasaki et al., 2007).

5.5. Spontaneous bioelectric activity as a dependent variable

Intrinsically generated spike trains in developing neural networks are exquisitely sensitive to the micro-environment in which they find themselves (e.g., Chang et al., 2006; Chiappalone et al., 2007; Golomb et al., 2006; Gramowski et al., 2004; Gross et al., 1992; Jun et al., 2007; Mao et al., 2001; Martinoia et al., 2005b), which under extreme experimental conditions can range from total cessation of firing – by blocking sodium and/or calcium action potentials or by blocking synaptic transmission (see Corner, 1994; Corner et al., 2002) – to maximal neuronal involvement in hyperexcited (Bausch et al., 2006; Ben-Ari and Holmes, 2006; Houweling et al., 2005; Le Bé and Markram, 2006; Milh et al., 2007) and/or disinhibited preparations: see Corner et al., 2002; Crain, 1976 for reviews; also Bosman et al., 2005; Corner et al., 2005, 2006; Khazipov et al., 2004; Wang and Xu, 2006. Especially intriguing is the recent report that, using selective glutamate receptor blockers, temporal patterning of spike trains in hippocampal cell cultures is more severely degraded by AMPA than by NMDA receptor blockade, whereas spatial characteristics such as synchronization and regularity of burst discharges depend more strongly on NMDA receptor activity (Li et al., 2007a).

Isolated cortical networks, which typically fire in a synchronized 'burst-pause' manner, can be induced to fire more or less tonically by exposure either to particular extra-cortical neurotransmitters (Chiappalone et al., 2007; Eytan et al., 2004; Tateno et al., 2005a,b) or to mild depolarizing conditions (Baker et al., 1991a; Jun et al., 2007; Leslie et al., 2001; also see Yvon et al., 2005). In addition, continuous firing – albeit with superimposed (midi-)bursts – emerges within minutes in organotypic cortex cultures when muscarinic acetylcholine receptors are blocked by atropine: (Corner et al., 2005). More generally, anything that reduces recurrent synaptic excitation along with activity-dependent outward membrane currents can trigger a transition from slow-wave sleep-like bursting towards a quasi-aroused tonic firing pattern (Compte et al., 2003).

Cortical networks can adapt quickly to milieu changes which affect their ongoing bioelectric activity. Thus, in immature rat hippocampal slices, pharmacological blockade of excitatory synaptic interactions leads well within an hour to depressed potassium currents, and thus to lowered thresholds for action potential induction (Van Welie et al., 2006). In rapidly developing but not in more mature organotypic visual cortex cultures, selective NMDA receptor blockade leads – after an initially profound depression – to recovery of control spontaneous firing levels within 24 h, with some neurons beginning to homeostatically adjust their spike discharge rates already within an hour whereas others take several hours to show the first signs of an increase (Corner et al., 2002). Subsequent acute blockade of AMPA receptors, on top of the blocked NMDA receptors, has revealed that it is this glutamate receptor sub-type that now provides all or most of the excitatory drive in these cultures. Simultaneous combined blockade of both AMPA and NMDA receptors in otherwise untreated cultures quickly results in total cessation of spontaneous spiking, which proved to be permanent over several weeks of culture in the presence of the blocking agents. Occipital cortex co-cultures (Baker and van Pelt, 1997), on the other hand, recover their original firing levels within a few days even with both the AMPA and the NMDA receptors blocked, and this time it is the kainate receptors which mediate the excitatory drive (Corner et al., 2005). During a restricted period in early ontogeny, tonic kainate receptor activity *in vitro* facilitates spontaneous glutamatergic synaptic currents in hippocampal (CA3) pyramidal cells, while reducing their incidence both in local interneurons and in downstream target cells (Lauri et al., 2005, 2006). If this finding also applies to normally developing neocortex explants, this might explain why we have never observed any acute effects of selective kainate blockade on mean action potential discharge rates in our own experiments (Corner et al., 2005).

Even a triple cocktail of pharamcological blockers, selective for the three ionotropic glutamate receptor sub-types, is unable to suppress firing for more than a few days in occipital cortex co-cultures. Acetylcholine receptors (muscarinic but not nicotinic) take over as the main source of excitatory synaptic interactions

within the network (see Section 2). Such a replacement of glutamate by acetylcholine as the major excitatory neurotransmitter, after the former had been chronically inactivated, was already on record for primary cultures of hypothalamus and spinal cord but not for neocortical neurons (Belousov et al., 2001, 2002). Indeed, it was only when we allowed two organotypic explants to cross-innervate one another (Baker and van Pelt, 1997) that cholinergic plasticity manifested itself in occipital cortex tissue. Although addition of the selective cholinergic antagonist, atropine, to the excitatory receptor suppressant 'cocktail' kept spontaneous firing rates from fully attaining control levels, even after several weeks in culture, it failed to prevent an as yet unknown source from in large measure compensating for even this drastic an interference with normal excitatory neurotransmission (Corner et al., 2005). Chub and O'Donovan (1998) have reported that, in chronically blocked developing spinal cord cultures, inhibitory GABA and glycinergic networks gradually resume the excitatory role normally played by glutamate and acetylcholine in mature networks, but the consistently negative results from our bicucculine assays make this highly unlikely in the case of the neocortex (Corner et al., 2005).

Tetrodotoxin reversibly eliminates all spontaneous action potentials in developing cerebral cortex cultures but, in relatively mature hippocampal cell cultures, the frequency of NMDA receptor-mediated miniature synaptic potentials and the incidence of functionally connected neuron pairs has been reported to increase significantly within 48 h (Nakayama et al., 2005). Subsequent reactivation in control medium has consistently revealed a strong compensatory up-regulation of excitatory mechanisms, expressed as enhanced spontaneous transmitter release (Murthy et al., 2001) as well as abnormally intense glutamatergic synaptic currents and/or high spike firing rates (reviewed in Turrigiano and Nelson, 2004 as well as in Corner et al., 2002; since then, see: Aptowicz et al., 2004; Bausch et al., 2006; Copi et al., 2005; Corner et al., 2006; Mateos et al., 2007; Nakayama et al., 2005). The rapid TTX-induced reduction in the frequency and amplitude of miniature inhibitory synaptic currents in developing cortical cell cultures (Colin-Le Brun et al., 2004; Kilman et al., 2002; Swanwick et al., 2006a) would only serve to potentiate these effects. Intracellular calcium levels, in some cases apparently acting as a monitor of transmembrane polarization, has been implicated in these and many other developmental neuroplasticity phenomena (e.g., Konur and Ghosh, 2005; Spitzer et al., 2002; Thiagarajan et al., 2002, 2005; Vanhoutte and Bading, 2003; West et al., 2001; Xiang et al., 2007). An especially striking example of early homeostatic plasticity involves the recovery, in late fetal rodent neocortex explants, from a week-long suppression of synchronous spike-driven calcium currents that would normally disappear within a week after birth: TTX-treated cultures, namely, continue to exhibit such activity for at least a week following transfer to control medium (McCabe et al., 2006).

Hyperpolarization of individual hippocampal neurons by means of experimental overexpression of potassium channels leads gradually, after an initial large reduction in the incidence of excitatory synaptic currents, to a homeostatic restoration of control firing levels in the transfected cells, and to a threefold further increase after several days of growth in the presence of TTX (Burrone et al., 2002). In a similar vein, the glutamate release probability from 1-week-old autaptic hippocampal synapses is enhanced following chronic treatment with either TTX or the selective NMDA receptor blocker, AP5; chronic AMPA receptor blockade had no noticeable effect in this experiment (Chavis and Westbrook, 2001). Several days of exposure to either TTX or the AMPA receptor blocker, DNQX, also leads to augmented spontaneous spiking in some inhibitory neocortical interneurons, due not only to intensified excitatory synaptic input but also to an increase in intrinsic cellular excitability (resulting from a weakening of transmembrane potassium currents: Gibson et al., 2006). Homeostatic network mechanisms can apparently take into account, as it were, not only the net level of ongoing cellular activity but also the balance between excitatory and inhibitory settings (Buckby et al., 2006; Karmarker and Buonomano, 2006). In analogous fashion, experimental elimination of spontaneously depolarizing GABAergic currents in vivo interferes drastically with not only inhibitory but also excitatory synaptic integration of newly born embryonic stem (ES) cell-derived granule neurons into the adult dentate gyrus (Ge et al., 2006, 2007; Karten et al., 2006). On the other hand, ES neurons cultured together with neonatal neocortex cells show the predicted augmentation of glutamatergic excitatory synaptic currents following a week-long exposure to TTX (Copi et al., 2005).

Combined AMPA and NMDA receptor blockade for one week in organotypic hippocampal cultures results upon release in vastly augmented amplitudes, durations and frequencies of excitatory post-synaptic potentials, while maintaining control levels of inhibitory activity (Buckby et al., 2006). The same treatment in 1–2-week-old rodent neocortex slice cultures leads initially to a total cessation of extracellularly recordable action potentials which, as with TTX, persists throughout the treatment period in isolated explants; subsequent assay in control medium after 2–3 weeks reveals the expected hyperactivity (see Corner et al., 2002). In bilateral cortical co-cultures, in contrast, firing levels and patterns are largely compensated for within 24 h whether NMDA receptors are blocked selectively or together with the AMPA receptors (Corner et al., 2005). As reviewed in Section 2 of this monograph, even much more drastic blockades of excitatory synaptic receptors fail to prevent the return of abundant synchronized burst discharges within a few days at most. This first-order homeostatic response of the developing network turns out to have a secondary dimension which, of course, becomes manifest only when the preparations are returned to control growth medium: with little exception, chronically treated explants show little or no sign of hyperactivity. Indeed, SAP firing levels may even be reduced (Corner et al., 2006; reviewed at length in Section 3, including an abundance of previously unpublished data).

One would have expected that even if, by virtue of the homeostatic restoration of spontaneous spiking, the inactivated glutamate receptors had been protected from up-regulating (and inhibitory interneurons from declining in number and/or efficacy) during the treatment period, they still ought to have caused excessive spontaneous firing when acting in synergy with the up-regulated non-blocked receptors that earlier had mediated functional recovery. Their failure to do so suggests that, in accordance with a 'Hebbian-style' weakening of inactive or ineffective synapses (Bi and Poo, 1998; Butts and Rokhsar, 2001; Butts et al., 2007; Dan and Poo, 2004; Desai, 2003; Fiumelli and Woodin, 2007; Goldberg et al., 2002; Reiter and Stryker, 1988; Sun, 2007; Turrigiano and Nelson, 2000, 2004; Van Rossum et al., 2000), the blocked excitatory receptors must actually have suffered down-regulation in 'forward reference', as it were, to an eventual restoration of normal physiological conditions. This prediction was able to be confirmed experimentally (see Section 3.1 and Corner, 2008), which further underscores the many levels of complexity involved in homeostatic functional mechanisms in the developing cerebral cortex.

Enhancement of normal ongoing bioelectric activity during early development can trigger an opposite homeostatic response from that produced by diminished activity. Thus, paroxysmal bursting elicited by chronic GABAergic disinhibition in cultured neocortex leads to a gradual reduction over several days in the incidence and intensity of both spontaneous bursts and background spiking

(Corner et al., 2005; Turrigiano and Nelson, 2004). Once returned to normal medium, however, chronically disinhibited neocortical cell cultures display reduced stereotyped network bursting in comparison with the untreated controls (Ramakers et al., 1991). This being the opposite homeostatic response to that induced by prolonged electrical silence (Corner and Ramakers, 1992; Ramakers et al., 1990), it reflects strengthened rather than weakened inhibitory synaptic activity (Ramakers et al., 1994; also see Corner et al., 2002). [There are indications that such up-regulation of GABAergic neurotransmission is in part mediated via the kainate sub-type of glutamate receptor (Lauri et al., 2005).] In a similarly homeostatic manner, either sustained AMPA receptor-mediated excitation or direct membrane depolarization in immature rodent hippocampal CA1 pyramidal cells triggers an unusually rapid compensatory decline in neuronal excitability (complete within 30 min), in this case as a consequence of augmented activity-dependent outward potassium currents (Van Welie et al., 2004) rather than of inhibitory synaptic activity. Epileptiform bursting, induced by low [Mg^{2+}] in organotypic hippocampal explants, disappears (along with a concomitant diminution of network excitability) already after an hour of continuous spontaneous paroxysmal activity (Gutiérrez et al., 1999).

The switch at an earlier stage of development from GABAergic synapses being excitatory (depolarizing) to being inhibitory is accelerated by increased GABAergic activation and, conversely, delayed by chronic receptor blockade (Fiumelli and Woodin, 2007; Ganguly et al., 2001; Ouardouz et al., 2006). Prolonged activation of GABA receptors in newborn rat parietal cortex slices, i.e., at a time when GABAergic transmission is still excitatory, triggers compensatory reductions in synaptic responsiveness (Heck et al., 2007). Paradoxically then, repetitive brief tetanic stimulation, mimicking spontaneous bursting in immature hippocampal slices *in vitro*, leads rapidly to a strong increase in the amplitude of spontaneous excitatory synaptic currents (by virtue of suppressing a kainate-mediated inhibition of glutamate release that disappears by itself in the course of normal development: Lauri et al., 2006). In this particular case, although the activity-dependent removal of a self-inhibitory mechanism leads to enhanced excitatory transmission, it nevertheless has a subtle homeostatic dimension to it by virtue of countering the tendency for immature glutamatergic synapses to allow only high-frequency spike bursts to propagate within the network (also see Lisman, 1997).

Chronic post-synaptic receptor activation for two days *in vitro* via brain-derived neurotrophic factor (BDNF) – a known intervening variable in several activity-dependent phenomena (Copi et al., 2005; Vanhoutte and Bading, 2003; Walz et al., 2006) – counteracts the decrease in GABA-mediated inhibitory synaptic currents, accompanied by intensified spontaneous action potential firing, which is induced in neocortical or hippocampal neurons by chronic tetrodotoxin exposure (Rutherford et al., 1997). In hippocampal cell cultures, prolonged BDNF application leads to a demonstrable enhancement of GABA release (Ohba et al., 2005; Yamada et al., 2002), with a resulting potentiation of inhibitory GABAergic synaptic currents (Swanwick et al., 2006a). Sustained direct depolarization of cortical neurons by graded increases in the extracellular potassium concentration gradually leads to a proportional compensatory reduction in excitatory synaptic currents (Leslie et al., 2001). In a similar vein, tonic electrical stimulation of organotypic neocortical explants for 2–3 days induces a prolonged decline in the incidence and intensity of burst discharges (Johnson and Buonomani, 2007), suggesting that the actual pattern of spontaneous spiking is not necessarily a major consideration in early neuronal plasticity (but cf. Nelson et al., 2002; Ramakers et al., 1991). This is a finding of considerable theoretical interest since tonic firing, during waking as well as REM sleep, has been postulated (Jouvet, 1972; Tononi and Cirelli, 2006, respectively) to exert the same homeostatic effects as those documented for slow-wave sleep-like burst discharges in a variety of *in vitro* model systems (reviewed in Corner et al., 2002; and the present monograph). Here again, mean activity levels and the relative timing of bursts (see Butts and Rokhsar, 2001; Lisman, 1997) rather than the precise details of spike patterning could constitute the main activity-dependent factor during early neural development.

5.6. Spontaneous bioelectric activity as an epigenetic factor

We have come a long way since the time when, almost half a century ago, intrinsically generated – i.e., 'non-reflexogenous' (Hamburger, 1963; Weiss, 1941) – bioelectric discharges in neural networks were still either unsuspected, ignored or dismissed as physiologically unimportant side-effects (discussed in Corner, 1994). The by now widely acknowledged ontogenetic importance of such 'spontaneous' activities commences, in fact, from the very onset of neuronal function (see Corner et al., 2002 for a thorough survey of the 20th century literature). For instance, reflecting what appears to be a widespread homeostatic principle in the developing central nervous system, a selective blockade of glutamate receptors (especially of the NMDA sub-type) reduces the survival and/or phenotypic maturation of GABAergic inhibitory interneurons in developing neocortex explants (De Lima et al., 2004, 2007), whereas chronic depolarization enhances the activity-dependent enhancement of their dendritic length and branching (Jin et al., 2003). Similarly, GABA-mediated depolarization of transplanted, embryonic stem cell derived, newborn granule cells is necessary for their structural maturation and incorporation into the adult dentate gyrus (Ge et al., 2006, 2007; also see Owens and Kriegstein, 2002).

In a complementary homeostatic manner, suppression of NMDA receptor activation in developing organotypic hippocampal cultures leads to an increase in the complexity of excitatory, viz., pyramidal cell, dendritic arborization (Luthi et al., 2001). At a still earlier stage, however, calcium channel activation or inhibition stimulates or depresses, respectively, the phenotypic differentiation of hippocampal pyramidal cells (Boukhaddaoui et al., 2001; Pieraut et al., 2007) as well as of neural progenitor cells derived from mouse cerebral cortex (D'Ascenzo et al., 2006). Similarly, chronic membrane depolarization accelerates spine formation in differentiating hippocampal neurons in culture (Sohya et al., 2007), while kainate receptor stimulation enhances presynaptic (filopodial) motility in younger cultures but reduces it in mature ones (Tashiro et al., 2003). In organotypic neonatal parietal cortex cultures, prolonged GABAergic depolarization is even able to augment the migration of differentiating neuroblasts (Heck et al., 2007). Immature cortical networks apparently need something of an activity-dependent 'push' before any homeostatic corrective mechanisms can come into play (also see below).

Spontaneous bioelectric activity in developing cortical neurons also has a pronounced influence on synaptogenesis. Hypoactive transfected hippocampal neurons form fewer excitatory connections during the initial phase of synaptogenesis *in vitro* but catch up later on, and eventually wind up with larger vesicle pools (Burrone et al., 2002). Similarly, neocortical cell cultures silenced with TTX display an initially slower rate of neurite outgrowth and synaptogenesis before eventually attaining permanently higher counts of excitatory dendritic spine synapses (Van Huizen et al., 1985, 1987a). Autaptic excitatory synaptogenesis in solitary neuron micro-cultures is also slowed down by early suppression of spontaneous spiking (Takada et al., 2005), as is the formation of perisomatic axon terminals and inhibitory synaptic boutons in

rodent organotypic neocortex cultures and intact brains (Chattopadhyaya et al., 2004, 2007). Conversely, NMDA receptor activation in developing neocortex networks induces new pre-synaptic release sites along with enhancement of vesicle turnover (Walz et al., 2006) while spontaneous action potential-driven glutamatergic excitation speeds up the establishment of functional connections between pairs of neurons (Le Bé and Markram, 2006) in acute rodent neocortical slices isolated at 2 weeks after birth, i.e., in the middle of the most rapid phase of new synapse formation (Van Huizen et al., 1985, 1987a,b). Neocortical synaptogenesis is also initially accelerated when SAP firing is chronically intensified by means of GABAergic disinhibition (a treatment which, additionally, advances the age *in vitro* at which 'exuberant' synapses begin to be pruned away: Van Huizen et al., 1987b).

Several days of complete electrical silence, brought about either by suppression of sodium action potentials or by non-selective blockade of glutamate receptors, causes an increase of mean synapse size and vesicle content in ~2-week-old hippocampal cell cultures (Murthy et al., 2001), and in pyramidal CA1–CA3 neurons of comparable age after a mere ~24 h of AMPA receptor blockade (Thiagarajan et al., 2005). Even 12 h of exposure to TTX appears able to produce a similar effect in very early (P3) postnatal cultures, along with a corresponding increment in several biochemical markers of neuronal differentiation and in AMPA-mediated miniature excitatory synaptic currents (Lauri et al., 2003). These homeostatic effects appear to be caused specifically by reduced NMDA receptor activation, since chronic AP5 treatment of neonatal hippocampal slice cultures for 2-weeks *in vitro* leads to a substantial increase in excitatory synapse counts and dendritic complexity of pyramidal neurons (Luthi et al., 2001). On the other hand, continued AMPA receptor-mediated glutamatergic activation is required for spine maintenance in mature slice cultures; otherwise, the synapses eventually become relocated *en masse* to dendritic shafts, and lose their post-synaptic membrane specializations (Mateos et al., 2007). Since suppression of transmitter release, but not of action potential generation, in such preparations has the same effect (which can be counteracted by AMPA application), miniature synaptic currents appear to exert enough of a trophic effect to stabilize spine morphology (McKinney et al., 1999). Spontaneous transmitter release is not sufficient to sustain all structural aspects of neural network formation, however, since suppression of spike-driven synaptic interactions leads to excessive neuronal death during early neocortical development (Baker et al., 1991b, 1992; Voigt et al., 1997).

By means of genetically manipulated potassium channel overexpression, a severe local reduction of early postnatal neocortical firing rates can also be achieved *in vivo*, a treatment which reduces terminal axonal branching on the part of the affected transcortical callosal projection neurons (which are excitatory: see Kumar and Huguenard, 2001; Baker et al., 2006), whereas a similar selective reduction of spontaneous firing in the contralateral target neurons is without effect on ingrowing axons (Mizuno et al., 2007). Layer and region specificity become reduced such that layer II/III somatosensory callosal axons invade layer I as well and, in addition, are no longer restricted to the primary somatosensory area (Wang et al., 2007). Ipsilateral GABAergic suppression of motor cortex function during a very narrow early postnatal period in the cat degrades the specificity of contralateral corticospinal projections *in vivo* but this can be corrected by subsequently allowing a return of activity while silencing the contralateral cortex (Friel and Martin, 2005, 2007). In the olfactory cortex, the development of pyramidal cell spine – but not shaft – synapses is reported to be dependent specifically on NMDA receptor activation during a brief postnatal time window (Poo and Isaacson, 2007). Here too, it looks as if spontaneous neuronal discharges facilitate cyto-morphological maturation during the earliest stages of ontogeny, and only later begin to exert homeostatic corrective effects (such as BDNF-mediated stimulation of inhibitory synapse formation in cultured solitary GABAergic interneurons: Palizvan et al., 2004).

A large amount of research continues to be devoted to activity-dependent post-synaptic receptor development in spontaneously firing *in vitro* 'model' systems (for a survey of earlier work, see Corner et al., 2002). The incorporation of AMPA receptors into synaptic membranes ('AMPA-fication': Voigt et al., 2005) is tightly time-locked to the appearance and intensification of spontaneous bursting in neocortex cell cultures, the suppression of which strongly reduces AMPA-fication during the several days that excitatory interactions are predominantly GABAergic in nature. In the continued presence of bicucculine, however, the AMPA-fication process resumes once synaptic excitation starts to become glutamate mediated, leading to control AMPA receptor levels being reached in less than a week (Voigt et al., 2005). If invading cortical axons arrive after the onset of spontaneous network bursting they are unable to contribute to the innervation of co-cultured sister networks, retracting and/or degenerating instead unless bioelectric activity is experimentally suppressed. This might mean that the pruning of exuberant spine synapses – c.q., numerical 'overshoot' – in electrically active untreated neocortex cell cultures (Van Huizen et al., 1985, 1987a,b; Van Ooyen et al., 1995) mostly affects the last of those synapses to form.

Suppression of glutamatergic excitation throughout the first week of hippocampal synapse formation *in vitro*, while leaving GABA receptors unaffected, leads to hyperactivity associated with larger and more numerous post-synaptic glutamate receptors (Buckby et al., 2006). The complexity of homeostatic adjustments during hippocampal development, however, is illustrated by the finding that 48 h of TTX-induced spike suppression in mature cell cultures causes a large increase in the number of functional NMDA (at the expense of AMPA) receptors, while the return of spontaneous firing initiates a phase of renewed AMPA receptor incorporation into synaptic membranes (Nakayama et al., 2005; also see Kiyosue et al., 2004). [Interestingly, in an analogous manner, inhibitory synapses in the neonatal rat superior colliculus require glutamate receptor stimulation in order to mature from glycinergic to GABAergic: Meier et al., 2002.] Micro-cultured solitary GABAergic hippocampal neurons, too, fail to form mature AMPA receptors (Rao et al., 2000), presumably owing to auto-suppression of their intrinsic bioelectric membrane currents. In contrast, 1–2 days of deprivation in 1-week-old visual cortex cell cultures, far from interfering with the structural and functional development of AMPA receptors, actually favors them (Wierenga et al., 2005).

Suppression of spontaneous neuronal firing for 48 h in developing hippocampal cell cultures, whether by TTX or by APV-induced NMDA receptor blockade, leads to abnormally small, structurally poorly differentiated and hypoactive GABA receptors (Swanwick et al., 2006a). Similar deprivation in neocortex cell cultures has the same effect (Kilman et al., 2002). In contrast, GABA receptors on solitary pyramidal cells in micro-culture develop normally despite the absence of extrinsic innervation, but are frequently found to be co-innervated together with NMDA receptors by a single autaptic axon terminal (Rao et al., 2000). In this case, it would appear that, by assisting in the maturation of inhibitory input channels, glutamatergic self-stimulation may be operating homeostatically to counteract potentially run-away excitation. Indeed, 2 weeks of continuous hyperactivity, induced by blocking either potassium channel or inhibitory synaptic activity, enhances the structural maturation of GABA receptors

in the pyramidal cell layer of developing hippocampal slice cultures (Holopainen and Lauren, 2003; Marty et al., 2004). In complementary homeostatic fashion, chronic AMPA receptor blockage had precisely the opposite effect.

5.7. New dimensions for further exploration

As documented above, much is now known about the intrinsic sources which drive neuronal action potential generation, and about the forces which modulate, restrict and ultimately terminate the network bursts which are repetitively triggered in developing neural networks. Still, many questions remain concerning the quantitative details, underlying mechanisms and ontogenetic significance of spike-train patterning on multiple time-scales (discussed in Agrawal et al., 2001; Basu and Liljenstrom, 2001; Butts and Rokhsar, 2001; Corner et al., 2002; Crochet et al., 2005, 2006; Geisler et al., 2005; McCormick, 1999; Silberberg et al., 2005; Whittington et al., 2000;). Since recent empirical and theoretical studies have amply documented the importance of ongoing 'background' activity for establishing the nature as well as the reliability of neuronal responses to incoming signals (Arieli et al., 1996; Brunel and Hansel, 2006; Chao et al., 2005; Crochet et al., 2005, 2006; Fellous et al., 2003; Fellous and Sejnowsky, 2003; Fox et al., 2006; Grinvald et al., 2003; Léger et al., 2005; Li et al., 2007a; Petersen et al., 2003; Sachdev et al., 2004; Sasaki et al., 2006; Shu et al., 2003a), the significance of this aspect of developmental electrophysiology can no longer afford to be overlooked.

Especially intriguing is the finding that synchronized high-frequency firing of neocortical inhibitory interneurons (in the beta/gamma range) controls the probability as well as the timing of action potential generation in pyramidal cells, and thus strongly modulates the intracortical spreading of excitation (Hasenstaub et al., 2005). Burst firing in particular has recently been postulated to preferentially drive those target neurons the intrinsic post-synaptic oscillatory membrane tendencies of which match intra-burst firing frequencies in the presynaptic input (Izhikevich, 2002; Izhikevich et al., 2003; Sasaki et al., 2006; Villacorta and Panetsos, 2005). Spontaneous bioelectric discharge levels appear to be able throughout life, furthermore, to determine the vector of activity-dependent plasticity, thus acting as a 'meta-plasticity' factor favoring potentiation over depression when background firing is strong and the reverse when it is weak (e.g., Castro-Alamancos et al., 1995; Milner et al., 2004; Speechley et al., 2007; Xiao et al., 1995). Perhaps surprisingly, high levels of ongoing neuronal activity can, in addition, reduce the ability of stimulation to induce any plastic reaction at all (Crochet et al., 2006).

As part of a general program of extrapolating cellular and network data to system and organism levels (e.g., Corner, 1994; Di Cristo, 2007; Grillner et al., 2005; Peng et al., 2007), it should be kept in mind that network plasticity becomes biologically meaningful only in so far as it affects the levels and patterns of action potential outflow to downstream target structures. It is therefore to be welcomed that a number of recent experimental studies have been devoted specifically to measuring the electrical activities of neocortical output neurons (Christophe et al., 2005; Golomb et al., 2006; Kerr et al., 2005; Kozloski et al., 2001; Kumar and Huguenard, 2001; Mao et al., 2001; Mizuno et al., 2007; Zhang, 2004). In order for ontogenetically induced changes in neural network outflow to be physiologically meaningful, however, they should also produce a significant modification of responsiveness in downstream structures which, in turn, must be reflected in the next tier of downstream targets, and so forth until a demonstrable alteration in motor output from the nervous system as a whole results. Conversely, the neurological source of an experimentally induced efferent abnormality could be more readily pinpointed, ontogenetically as well as neurophysiologically, when central nervous subsystems are grown, treated and analyzed separately from one another. A highly promising development towards this goal is the use of compartmentalized culture chambers in which selected groups of neurons are accessible to independent experimental manipulation and recording (e.g., Ben-Ari, 2006b; Claverol-Tinturé et al., 2005, 2007; Fields et al., 1991; Jun et al., 2007; Vogt et al., 2005; Wyart et al., 2002; Yvon et al., 2005).

An exceedingly important final consideration in regard to the relevance of experimental findings for understanding normal functioning in intact organisms is the physiological state of the 'model' system used for the study. Demonstrable functional abnormalities during the (slow wave) sleep-like condition characteristic for neuronally isolated cortical networks need not, for instance, have any noteworthy counterparts when the cortex is in an 'aroused' state corresponding to wakefulness or 'paradoxical' sleep (see discussion in Sections 5.1 and 5.5). A distinction between these latter two states is a further *sine qua non* for adequate assessments to be made of the putative behavioral relevance of abnormal brain activities measured in an experimental context. Here too, the advantages of selective electrical and chemical access to each component of a model system will be readily apparent, with respect not only to screening for activity-dependent developmental defects, but also to exploring possibilities for reversing ontogenetically rooted abnormalities using targeted interventions.

A fascinating question in this regard concerns theoretical possibilities for altering structural and functional settings which, once established during ontogeny, operate so as to defy subsequent therapeutic protocols for modifying undesirable patterns of activity (but see Friel and Martin, 2007; Martin et al., 2007). Little is known as yet, however, about the undoubtedly highly complex processes whereby a multiplicity of homeostatic set-points, in concert dictating the brain's operational steady-states, become programmed into the developing nervous system. Given such restorative tendencies, the large inter-individual variations which are often seen among purportedly identical preparations, even when coming from the same donor animal, is especially puzzling. This can only mean that the set-point tendencies themselves, or else the homeostatic capacity to realize them, are not programmed into the developing neurectoderm in a homogeneous manner. Indeed, already at the neurula stage, a extremely broad scala of regionally non-specific activity levels and patterns emerges when small pieces of central nervous tissues are cultured (Corner, 1964a). Only when large enough pieces of neural plate are allowed to develop in isolation do reproducible, regionally specific, spontaneous action patterns become the rule. Also in organotypic neocortex cultures, isolated long after region-specific brain differentiation has become determined (see Nieuwkoop et al., 1985), 'mega' cultures embracing many cortical areas exhibit far less culture-to-culture variance than do smaller, area-specific explants (Baker et al., 2006). One may presume that, owing to regulatory propensities activated by regional interactions, local developmental programs become modified and entrained so as to form a restricted set of spatially well-defined basins of attraction (Goodwin and Cohen, 1969).

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