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CHAPTER 18

Compensatory physiological responses to chronic blockade of amino acid receptors during early development in spontaneously active organotypic cerebral cortex explants cultured in vitro

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Abstract: Paired organotypic explants from rat occipital cortex were cultured for up to three weeks in the presence of selective blockers of amino acid receptor blockers, during which period spontaneous action potential generation was monitored electrophysiologically. In contrast to isolated explants (Corner, M.A., van Pelt, J., Wolters, P.S., Baker, R.E. and Nuytinck, R.H. (2002) Physiological effects of sustained blockade of excitatory synaptic transmission on spontaneously active developing neuronal networks — an inquiry into the reciprocal linkage between intrinsic biorhythms and neuroplasticity in early ontogeny. Neurosci. Biobehav. Rev., 26: 127–185), which upregulated their initially depressed spontaneous bursting activity only under conditions of N-methyl D-aspartate (NMDA) receptor blockade, cross-innervated co-cultures showed a large degree of functional recovery even when combined NMDA and AMPA receptor blockade was carried out. This compensatory activity could be eliminated by acute addition of a selective kainate receptor blocker to the medium. When kainate along with AMPA and NMDA receptor mediated activity was *chronically* suppressed, however, considerable functional recovery — in the form of recurrent burst discharges — took place gradually over a period of three weeks in vitro. These spontaneous bursts disappeared rapidly upon treatment with the muscarinic receptor blocker, atropine, but continuous low-level firing emerged at the same time. Similar ''tonic'' background activity was induced in control cultures as well, but without any noticeable reduction in burst discharges. Co-cultured neocortex explants, in which cyto-morphological maturation proceeds to a far greater degree than in isolated explants (Baker, R.E. and van Pelt, J. (1997) Co-cultured but not isolated cortical explants display normal dendritic development: a longterm quantitative study. Dev. Brain Res., 98: 21–27) are evidently capable of an astonishing degree of functional compensation for loss of excitatory synaptic drive during development. It could be shown, furthermore, that such homeostatic responses are not mediated largely by a weakening of inhibitory mechanisms in the absence of spontaneous firing. Chronic inhibitory synaptic blockade, on the other hand, led to intensified bursting activity which gradually normalized over a 3-week culture period. The cellular basis for this reversal of the disinhibited state, as well as for the residual neuronal firing even after cholinergic mechanisms have been largely eliminated, is at present unknown. The degree to which immature cortical networks attempt to compensate for altered levels of physiological activity, as documented in the present report, is another indication of how important such activity can be for normal development (see Corner, M.A., van Pelt, J., Wolters, P.S., Baker, R.E. and Nuytinck, R.H. (2002) Physiological effects of sustained blockade of excitatory synaptic transmission on spontaneously active developing neuronal networks — an inquiry into the reciprocal linkage between intrinsic biorhythms and neuroplasticity in early ontogeny. Neurosci. Biobehav. Rev., 26: 127–185). At the same time, the large variations in overall firing levels and ''macro-scale'' temporal patterns from culture to culture within a given series, despite all attempts at identical preparation of the explants, can only mean that the ''set-points'' for such regulation are themselves subject to

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unknown ontogenetic factors which, apparently, are nonuniformly distributed even within a restricted region of the neocortex. On the other hand, it was striking to note that, regardless of age or treatment, an unexpected degree of consistency in temporal patterning existed at "mini-" and "micro-" time-scales (viz., EEG *delta* and *beta* frequency ranges, respectively) even when network bursting tendencies became greatly reduced in favor of tonic firing.

Introduction

Complex quasi-rhythmical firing originating within neural networks themselves has been demonstrated to be not only a widespread phenomenon in the developing nervous system but to exert a considerable influence on its structural and functional maturation (for review see Corner et al., 2002). The effect of chronically blocking spontaneous activity at early stages is, in general, to induce a state of hyperexcitability characterized by intensified, often quasi-epileptiform neuronal bursting patterns. Diminished inhibitory synaptic drive has been demonstrated to underlie this phenomenon in several tissue culture "model" systems, but various other homeostatic mechanisms have been implicated as well (also see Davis and Bezprozvanny, 2001; Burrone and Murthy, 2003). Isolated "organotypic" explants prepared from neonatal rat occipital cortex, moreover, eventually developed hyperexcitability even when only the NMDA receptors were chronically blocked, despite the fact that the treated cultures displayed high levels of spontaneous network burst activity (SBA) throughout most of their development in vitro. Indeed, such explants became even more active upon return to normal medium than did those in which SBA had been eliminated completely by chronic blockade of the AMPA along with the NMDA receptors. Paradoxically, selective blockade of the former led to a strong *reduction* of spontaneous discharges when assayed in control recording medium following several weeks of treatment.

Quantitative cyto-morphological studies have since revealed that little or no elaboration of dendritic arborization takes place in isolated neocortical explants, in striking contrast to the practically normal neuronal branching patterns which develop over several weeks in paired co-cultures (Baker and van Pelt, 1997). Such preparations become extensively cross-innervated, which presumably provides a source of trophic stimulation to the developing neurons. The effect of cross-innervation could not be duplicated either by simply enlarging the size of isolated explants or by co-culturing them in an orientation (e.g., pia-to-pia) which did not allow the outgrowing neurites from one explant to extensively penetrate the other. Spontaneous firing patterns, too, failed to show the characteristic maturational changes described earlier for dissociated neocortical cell cultures, and persevered in the primitive quasi-paroxysmal network bursting mode (see Corner and Ramakers, 1992). In view of the morpho-physiological deficiencies thus brought to light in this "model" system, along with the demonstrated importance of SBA in early ontogeny, it was decided to examine the neurophysiological consequences of chronic blockade of amino acid receptors in cross-innervated co-cultures. The results to be presented here will show that not only did considerable functional maturation take place, but also that the spontaneous discharges recorded in this preparation have given the best approximation to the firing patterns of intact developing neocortex (Mirmiran and Corner, 1982) yet observed under in vitro conditions. More importantly, it turns out that the capabilities of developing neural tissues to "homeostatically" compensate for persistent interference with synaptically mediated communication within the network (cf. Corner et al., 2002) is far greater and more complex than hitherto suspected.

Methodological considerations and procedures

Organotypic explants of neonatal rat occipital cortex were prepared as described previously (Baker and van Pelt, 1997) and cultured in vitro for one to three

weeks, at which time they were monitored for spontaneous bioelectric activity as described in Corner et al. (2002). Cultures were treated chronically with one or a combination of amino acid receptor blockers in supramaximal doses, and were recorded in their respective growth media so as to chart the course of recovery from changes induced by acute exposure to the same agents. Since little or no "adaptation" to the experimental setup was observed to be needed before an explant settled into a stable firing pattern, electrophysiological recording (from cortical layers II–IV: see Baker and van Pelt, 1997) typically commenced within 10 min after transfer of the culture dish from the incubator. Polyneuronal spike train registration, using saline filled glass micropipettes of ca. 10 μm diameter, continued uninterrupted for ca. 15 min, during which time all action potentials exceeding twice the baseline noise level were stored as time-stamps for off-line analysis. Media changes were effected without disturbing the ongoing recording, and the resulting spike-trains were transferred to separate computer files starting from the time that a new stationary firing pattern appeared on the time plots (typically within 10 min). If spontaneous activity ceased, as it did on rare occasions, the explant was probed before discontinuing the experiment to see if another active site could be found. Only a single site per explant was used for statistical analysis.

Time-stamps were computer analyzed using an in-house developed program: "jspike" (for sample plots and raw traces and for details of the procedure, see Corner et al., 2002). Besides the overall mean firing rate and its minute-to-minute variance, this program calculates a variety of bursting parameters for *ad libitum* selected "burst" criteria. Based upon the preferred location of troughs separating relatively short from longer intervals in the interval histograms, a criterion of 1 s was adopted in order to define the incidence of recurrent "network bursts" (this term was adopted since previous studies — e.g., Crain, 1976; Van Pelt et al., 2004 — have shown that they appear in synchrony throughout the entire excitable system). In addition, an interspike interval criterion of 10 s was adopted in order to quantify the tendency for such bursts to be clustered in trains of variable duration, separated by long periods of nearly total quiescence (Corner et al., 2002). Similarly, a criterion of 100 ms was used for quantifying the tendency for each of these network bursts to be composed of still shorter clusters of action potentials at intervals of several hundred milliseconds: these latter will henceforth be referred to as "*mini-bursts*". These proved to be made up, in turn, of brief trains of still shorter spike clusters which we will refer to as "*micro-bursts*". For the analysis of mini- and micro-burst activity, maximum permissible interspike intervals of 1000 and 100 ms, respectively, were introduced in order to ensure as accurate as possible a hierarchical picture of spike clustering tendencies on successively finer timescales.

Using the above-mentioned criteria, the mean values for the following parameters were computed for each spike train: burst *duration* and its coefficient of variation (listed as "*variance*" in the tables), number of spikes ("*count*") within each burst; *intensity* of firing during bursts, and the time elapsing between the onset of successive bursts ("*period*"). Since long interspike interval criteria yielded "*burst ratio*" values (i.e., the proportion of spikes which occurred during a burst, as defined by a given criterion) approaching 100% in practically all age and treatment groups, this ratio was defined using a criterion of 100 ms, since this choice enabled statistically significant differences in the tendency toward burst behavior to be demonstrated. Finally, the *modal* interspike intervals for individual recording sessions were estimated on the basis of log–log plots of the interval histograms. For each experimental group, the median and 50% quartile values were calculated for all of the selected parameters, and these were compared with one another using nonparametric statistics. The Kruskal–Wallace, Chi-square and Bonferroni tests were employed as a preliminary "analysis-of-variance", after which pair-wise group comparisons were made using the Mann–Whitney, Chi-square and Fischer exact-probability tests. In experiments where spontaneous firing patterns before and after addition of pharmaca were compared, either the Wilcoxon matched-pairs, signed-ranks or the Sign test was used.

Chronic experiments entailed addition of the following pharmaca to the growth medium:

(a) *bicuculline*, in order to suppress GABAergic inhibitory synaptic drive;

(b) DL-2-amino-5-phosphonopentanoic acid (*apv*), for blocking NMDA glutamatergic receptors;
(c) 6,7-dinitroquinoxaline-2,3-dione (*dnqx*), for blocking AMPA glutamatergic receptors; and
(d) *apv* + *dnqx*, in an attempt to totally suppress excitatory synaptic drive (see Corner et al., 2002).

When it turned out that spontaneous activity gradually returned even in this last preparation, in striking contrast to isolated cortical explants (Corner et al., 2002), a separate series of experiments was run in which the glutamate blocker "cocktail" (i.e., group d) was supplemented by "LY382884", a specific blocker of kainate receptors (courtesy of Eli Lilly & Co, Indianapolis, USA). Since partial recovery still took place in this LAD cocktail, the cholinergic/muscarinic blocking agent *atropine* was added in some cases, in an attempt to pinpoint the source of the emergent excitatory activity. Acute additions of the other pharmaca (viz., a–d) were also sometimes made in the course of the chronic experiments, as a means of identifying changes at the receptor level which might account for the return of function.

Functional development in the presence of selectively blocked amino acid synaptic receptors

Chronic treatment for 1 week in vitro

At 1-week in vitro, bicuculline treated cultures showed shorter but more intense "network bursts" than control cultures (Fig. 1; Table 1: 1000 ms interspike interval criterion), as expected on the basis of the acute effect of this GABAa receptor blocker (data not shown, but see Corner and Ramakers, 1992). At the micro- and mini-levels (i.e., 10 and 100 ms criteria, respectively) spontaneous bursts of activity in these chronically disinhibited cultures lasted longer than in control cultures, and also the sequences of network-burst trains — i.e., the "macro-" level of activity — were enormously prolonged (Fig. 1; also see Table 1, 10000 ms criterion: duration). APV permitted a virtually complete recovery of normal

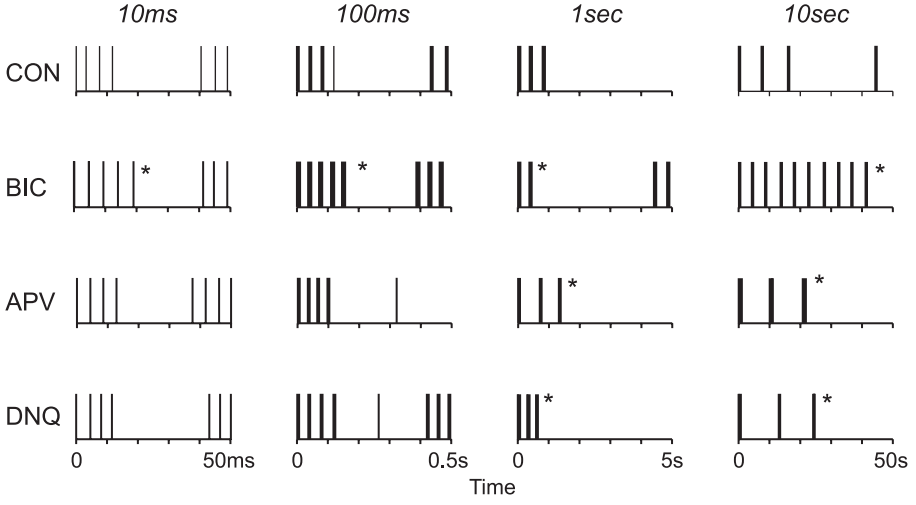

Fig. 1. Reconstruction of "modal firing patterns" in explants cultured for 1-week in the presence of different amino acid receptor blockers and recorded in their respective growth media. Four different time-scales are shown for each group, corresponding to the different interspike intervals used as a criterion for defining clusters, c.q. "bursts", of spontaneous action potentials. These "caricatural" plots were derived from the modal values in Table 1 — to which the reader is referred for exact parameter values — for the *duration, count* and *period* calculated for each of the intervals used as a burst criterion (see top of each column). An asterisk at the end of a spike cluster indicates a difference ($p < 0.05$) from control values for either the duration, count or firing intensity of the burst, while an asterisk at the end of an interburst interval indicates a significant difference in periodicity. The occasional presence of an isolated spike in between successive bursts is dictated by the "*burst ratio*" parameter (see Table 1). CON = controls; BIC = bicuculline; DNQ = dnqx treated cultures.

Table 1. Spontaneous activity as recorded in growth media containing selective amino acid receptor blockers: neuronal firing patterns after 1-week of culture in vitro (the numbers of cultures analyzed are given in parentheses)

Group	Control (23)	Bicuculline (13)	APV (27)	DNQX (22)
MFR (sp/s)[a]	1.47–**1.97**–3.46	3.15–**4.51**–5.99[c]	1.06–**1.62**–2.62[e]	1.03–**1.39**–1.89[ce]
Modal ISI (ms)[a]	4–**7**–20	7–**9**–10[b]	8–**10**–65[cd]	8–**10**–27[cd]
Burst Ratio[a]	0.91–**0.96**–0.98	0.94–**0.98**–0.99	0.75–**0.91**–0.97	0.69–**0.84**–0.97[c]
CV-60 s (%)[a]	49–**62**–112	30–**45**–95[b]	70–**107**–129[bd]	35–**60**–80[g]
10,000 ms criterion				
Duration (s)[a]	10.2–**15.5**–20.0	22.7–**41.4**–122[c]	10.4–**22.9**–40.3[bd]	10.4–**26.3**–63.8[b]
Coeff. Var. (%)[a]	59–**76**–107	92–**115**–134[b]	78–**125**–183[c]	103–**126**–177[c]
Count (sp/bur)	50–**100**–243	256–**374**–983[c]	57–**120**–285[d]	70–**105**–284[d]
Intensity (sp/s)[a]	2.42–**4.90**–10.6	7.49–**9.24** -10.6[b]	2.45–**6.52**–10.6[d]	2.49–**4.61**–7.19[e]
Period (s/c)[a]	32.8–**44.0**–72.2	73.2–**90.8** –147[b]	57.5–**80.9**–132[b]	52.9–**66.5**–90.7
1000 ms criterion				
Duration (s)[a]	0.45–**0.95**–2.10	0.19–**0.36**–1.98[b]	0.98–**1.29**–3.18[be]	0.48–**0.86**–1.72[df]
Coeff. Var. (%)	117–**193**–239	144–**190**–225	178–**187**–250[f]	80–**159**–246
Count (sp/bur)	9–**25**–47	13–**27**–47	12–**22**–49	8–**13**–42
Intensity (sp/s)[a]	17.6–**24.9**–34.1	25.5–**61.6**–72.3[c]	8.79–**15.4**–27.4[bd]	9.82–**15.2**–40.6[bd]
Period (s/c)[a]	6.51–**9.41**–18.2	3.76–**4.67**–6.80[b]	5.56–**13.6**–35.0[e]	8.46–**12.2**–16.1[e]
100 ms criterion				
Duration (ms)	80–**126**–203	120–**145**–220[b]	53–**106**–179[d]	89–**128**–212
Coeff. Var. (%)[a]	110–**139**–209	61–**123**–198	68–**98**–165[c]	65–**81**–102[c]
Count (sp/bur)[a]	6–**9**–14	8–**17**–19[c]	6–**7**–11[d]	6–**9**–13[d]
Intensity (sp/s)	55.3–**67.1**–86.2	66.1–**75.6**–91.9	48.0–**71.4**–89.4	49.6–**57.9**–76.3[d]
Period (ms/c)[a]	361–**442**–538	318–**401**–460	453–**586**–665[ce]	272–**417**–513[g]
10 ms criterion				
Duration (ms)	10–**12**–17	11–**19**–21[b]	9–**12**–16[d]	9–**12**–16[d]
Coeff. Var. (%)	58–**72**–89	74–**97**–136[b]	66–**75**–101	77–**89**–123[bf]
Count (sp/bur)	3–**4**–5	3–**5**–6	3–**4**–5	3–**4**–5
Intensity (sp/s)	204–**226**–256	196–**203**–213[b]	211–**219**–246	196–**210**–225
Period (ms/c)	34–**41**–49	38–**42**–45	31–**38**–45	37–**43**–46

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 **mode** and the **50% quartile** values for the number of cultures given above in parentheses).
[a]overall *treatment* effect ($p < 0.05$); [b]$p < 0.05$, [c]$p < 0.01$ vis a vis the control group; [d]$p < 0.05$, [e]$p < 0.01$ vis a vis the bicuculline group; [f]$p < 0.05$, [g]$p < 0.01$ between the APV and the DNQX group.

firing levels following an initial ca. ten-fold reduction immediately following application of this selective NMDA receptor blocker (Table 2). [The time-course of recovery was not followed in the present experiments, but in isolated cortical explants it was found to commence within 1 h and to be complete within 24 h (Corner et al., 2002)]. In sharp contrast to APV, the only effect of acute DNQX administration was to cause a slight increase in network-burst durations and an increase in their incidence, along with greater minute-to-minute fluctuations in mean firing. None of these changes were in evidence in the *chronically* treated DNQX group, but the overall firing level remained somewhat depressed and the tendency towards bursting behavior was slightly reduced (Table 1). AMPA-receptor mediated synaptic drive is apparently quite weak in the neocortex at this stage of development (also see Corner et al., 2002). Acute selective kainate receptor blockade with LY had a stronger suppressant effect than expected, with activity levels about midway between those of APV and DNQX (see Table 2: MFR). Further, the tendency toward burst firing was weakened in the absence of kainate receptor function, while minute-to-minute fluctuations were greatly enhanced (an effect also noted upon acute APV addition to the medium: see table 2: CV-60 s).

Rather to our surprise, whereas acute exposure to this "cocktail" always led rapidly to complete cessation of spiking (data not shown),

Table 2. Acute effects of selective excitatory amino acid receptor blockade on spontaneous firing patterns in 1-week-old control cultures

Group	APV (7)	LY (11)	DNQX (9)
MFR (sp/s)[a]	0.13–**0.19**–0.44[cd]	0.45–**0.93**–1.60[d]	0.99–**1.39**–2.33
Burst Ratio	0.77–**0.91**–0.93	0.83–**0.90**–0.93[d]	0.84–**0.91**–0.95
CV-60s (%)[a]	192–**227**–238[cd]	97–**164**–229[d]	91–**97**–113[cd]
1000 ms criterion			
Duration (s)[a]	0.36–**0.56**–0.76[b]	1.20–**1.66**–2.54	1.22–**1.73**–2.15[b]
Coeff. Var. (%)[a]	105–**112**–196	160–**182**–244	113–**145**–203
Count (sp/bur)[a]	7–**12**–15[bd]	15–**31**–43	19–**36**–56[b]
Intensity (sp/s)	16.1–**18.7**–30.6	13.5–**19.4**–21.8[d]	14.7–**21.7**–31.5
Period (sec/c)[a]	11.1–**20.0**–30.1	14.0–**16.2**–29.1	16.4–**34.4**–49.0[cd]
100 ms criterion			
Duration (ms)[a]	54–**80**–118	138–**199**–294	98–**171**–300
Coeff. Var. (%)	101–**117**–140	99–**106**–142[e]	112–**128**–157[b]
Count (sp/bur)	4–**4**–8	7–**9**–16	7–**9**–17
Intensity (sp/s)[a]	66.0–**92.6**–99.1	42.7–**49.3**–57.1[d]	53.6–**57.8**–60.3
Period (ms/c)	405–**444**–461	434–**442**–503	444–**458**–508

Abbreviations as in Table 1.
[a]Overall *treatment* effect ($p < 0.05$); [b]$p < 0.05$, [c]$p < 0.01$ vis a vis the corresponding *chronically* treated group (see Table 1); [d]$p < 0.05$ vis 'a vis *control* values immediately prior to treatment.

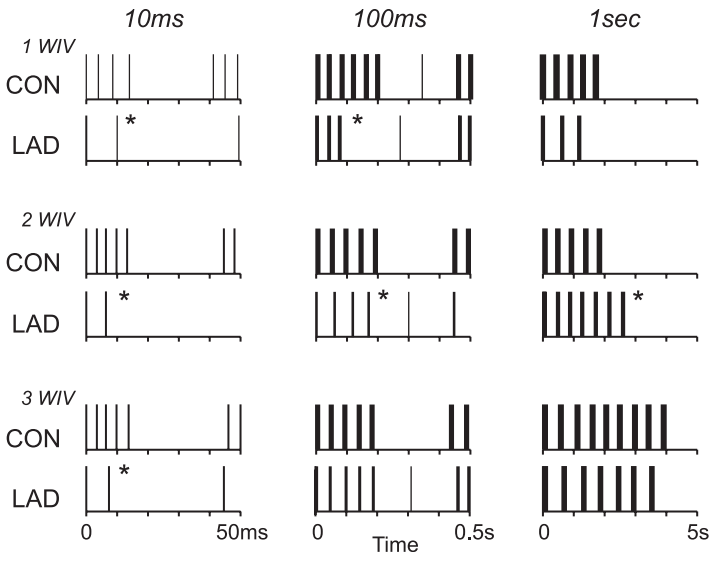

Fig. 2. Reconstruction of "modal firing patterns" in explants cultured in medium containing the "LAD cocktail" for simultaneously blocking glutamatergic AMPA, kainate and NMDA receptors for 1–3 weeks (see Methods section for full details). Explanations as in Fig. 1.

spontaneous activity was present in most of the "LAD"-treated 1-week-old cultures (see Methods section), albeit at a greatly reduced level (Fig. 2). Network-burst durations did not change noticeably under these conditions (Table 3: 1000 ms criterion) but mini- and micro-bursts were much shorter and less intense than in the controls (100 ms and 10 ms criteria, respectively). Also striking were

Table 3. Spontaneous firing patterns at 1- and 2-weeks in vitro during chronic blockade of AMPA, NMDA and kainate receptors (LAD group)

	One week		Two weeks	
Group	Control (20)	LAD (16)	Control (25)	LAD (22)
MFR (sp/s)[a]	1.75–**2.52**–3.41	0.38–**0.54**–1.42[cd]	3.08–**5.87**–7.21	0.14–**1.55**–2.84[be]
Modal ISI (ms)[a]	9–**10**–25	25–**185**–1000[c]	2–**3**–6	44–**70**–200[c]
Burst Ratio[a]	0.88–**0.93**–0.96	0.08–**0.54**–0.72[c]	0.97–**0.99**–0.99	0.49–**0.80**–0.85
CV 60 s (%)[a]	91–**102**–116	61–**85**–120[c]	50–**69**–82	53–**71**–88
1000 ms criterion				
Duration (s)[a]	1.19–**1.76**–2.61	0.86–**1.21**–1.92	0.24–**1.78**–4.45	1.34–**2.48**–9.47[c]
Coeff. Var. (%)[a]	166–**216**–271	85–**112**–127[c]	87–**119**–183	109–**149**–210
Count (sp/bur)[a]	23–**34**–45	3–**5**–11[c]	25–**73**–163	6–**28**–70
Intensity (sp/s)[a]	16.2–**24.6**–30.7	2.16–**3.75**–5.72[c]	35.1–**51.8**–71.3	3.99–**8.30**–10.6[c]
Period (s/c)[a]	9.81–**10.9**–20.4	6.79–**7.99**–14.0[c]	5.72–**10.6**–26.1	9.21–**13.5**–21.7
100 ms criterion				
Duration (ms)	161–**204**–287	43–**86**–127[c]	157–**202**–290	67–**142**–196[b]
Coeff. Var. (%)[a]	118–**152**–180	54–**88**–140[c]	71–**166**–212	75–**92**–201
Count (sp/bur)[a]	10–**13**–19	2–**3**–3[c]	13–**21**–27	3–**4**–6[c]
Intensity (sp/s)[a]	54.9–**60.3**–69.8	21.9–**25.5**–33.0[c]	79.4–**104**–116	20.3–**21.0**–27.9[c]
Period (ms/c)	443–**456**–528	459–**477**–499	414–**456**–529	404–**445**–461
10 ms criterion				
Duration (ms)[a]	11–**13**–15	7–**9**–10[c]	13–**16**–20	6–**7**–8[c]
Coeff. Var. (%)	92–**104**–116	61–**75**–90[c]	78–**97**–122	39–**50**–60[c]
Count (sp/bur)[a]	4–**4**–5	2–**2**–3[c]	5–**5**–6	2–**2**–3[c]
Intensity (sp/s)[a]	218–**225**–239	174–**184**–189[c]	244–**273**–301	166–**173**–191[c]
Period (ms/c)[a]	41–**42**–45	44–**50**–55	42–**45**–47	48–**56**–67[c]

Abbreviations as in Table 1.
[a]Significant *age* effect for the control cultures (cf. Table 9); [b]$p < 0.05$, [c]$p < 0.01$ vis à vis the corresponding control group; [d]includes 3 silent cases, [e]includes 6 silent cases which later became active in control medium.

the dramatic lengthening of the modal interspike interval and increase in the percentage of "isolated" spikes falling in between the mini-bursts (Table 3: *burst ratio*). Since increasing the dosage was ineffective in further suppressing SBA during chronic LAD treatment, it must be concluded that a non-glutamatergic source of excitatory drive had become operative. In view of the observation that *acute* blockade of all three glutamatergic receptor types was able to completely silence control cultures, this emergent novel source of excitation would appear not to normally influence SBA to any noticeable extent.

Chronic treatment for 2 weeks in vitro

Control cultures at 2 weeks in vitro showed little or no change at the micro- or mini-burst level from the situation at 1-week, but network-bursts had increased in duration by more than three-fold, thus resulting in long trains of mini-bursts at ca. 500 ms intervals (Fig. 3; Table 4: 100 and 1000 ms criteria). Mini-bursts in 2-week-old bicuculline treated explants changed little since the first week, but continued to be longer and more intense than in the corresponding controls. Network bursts, on the other hand, were now more than twice as long as they had been earlier, although still considerably shorter than in control explants (Fig. 3; also Table 4, 1000 ms criterion: *duration*). Owing to their reduced tendency to occur in long trains, however, overall firing rates in the bicuculline group had fallen to less than half the values measured at 1-week in vitro (Table 4: *MFR*). In addition, *modal* interspike intervals were significantly longer and showed much less variation from culture to culture than in the control group.

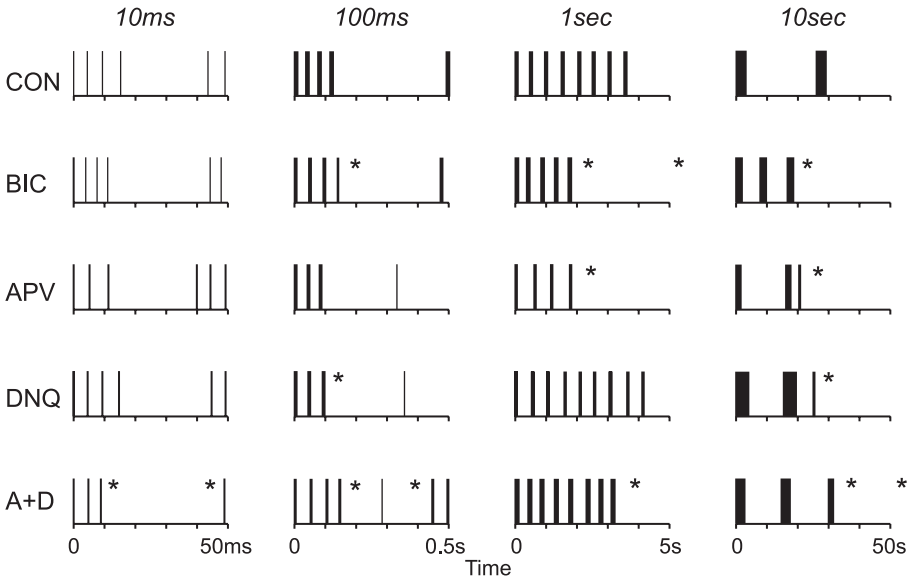

Fig. 3. Reconstruction of "modal firing patterns" in explants cultured for two weeks in the presence of different amino acid receptor blockers. Explanations as in Fig. 1.

Firing patterns in APV-treated explants were very similar to the chronic bicuculline induced patterns, except that mini-bursts were shorter and tended to occur at longer intervals (Table 4: 100 ms criterion). DNQX-treated explants were similar in this respect, too, but they differed from the other two experimental groups in that mini-burst trains lasted more than twice as long, on the average, thus approximating the network-burst durations seen in control cultures (Fig. 3). Explants treated with APV in combination with DNQX had significantly shorter than normal micro-bursts (Table 4: 10 ms criterion), which resulted in lower overall firing rates since all other burst parameters were comparable to the control values. It is nevertheless noteworthy that extreme minute-to-minute *fluctuations* (i.e., the CV-60 s measure), large variations in burst *durations*, abnormally long modal interspike *intervals*, and intensified "*background*" firing in between bursts (i.e., the *burst ratio*) were characteristic for this group of cultures (see Table 4).

Cultures subjected to chronic total blockade of glutamate receptors for 2 weeks (LAD group: see Table 3) were indistinguishable from APV + DNQX treated cultures except for a reduced tendency toward wild fluctuations on all time-scales. In other words, they had achieved partial recovery of overall activity since the level seen at 1-week in vitro, although both mini- and micro-bursts remained extremely weak (Fig. 2; also Table 3: 100 ms and 10 ms criteria, respectively).

Chronic treatment for 3 weeks in vitro

Explants kept for 3 weeks in vitro in a state of chronic disinhibition (viz. the bicuculline group) kept pace with the controls as far as their overall activity levels, modal interspike intervals and burst-ratios were concerned (Table 5). Their minute-to-minute firing rates (CV-60 s measure) were more stable, however, and their mini-bursts lasted more than twice as long on average than in control cultures (see Fig. 4), and showed a smaller variance among individual explants. Possibly owing to the strong intensification of (mini-)burst discharges, "network bursts" lasted no longer than they did a week earlier, so that they were now even shorter in comparison with control bursts than at 2 weeks in vitro. As at 2 weeks, the intervals between successive network bursts were considerably shorter in the bicuculline than in the control group (Fig. 4; and see Table 5,

Table 4. Spontaneous activity recorded in growth media containing selective amino acid receptor blockers: neuronal firing patterns after 2-weeks of culture in vitro (number of cultures in parentheses)

Group	Control (24)	Bicuculline (11)	APV (28)	DNQX (28)	APV + DNQX (23)
MFR (sp/s)[a]	1.09–**2.17**–7.2	1.38–**1.56**–3.33	1.06–**1.54**–2.31[bf]	1.10–**2.95**–5.66[gh]	0.74–**1.12**–2.03[cd]
Modal ISI (ms)[a]	6–**7**–8	9–**15**–20[b]	4–**8**–10[d]	4–**9**–23[bg]	7–**30**–93[cg]
Burst ratio[a]	0.85–**0.94**–0.98	0.79–**0.95**–0.96	0.88–**0.94**–0.99	0.83–**0.90**–0.96	0.64–**0.75**–0.94[cdg]
CV-60s (%)[a]	79–**98**–119	68–**81**–96	96–**129**–166[ce]	79–**98**–123[gh]	102–**129**–207[ce]
10,000 ms criterion					
Duration (s)[a]	14.7–**30.1**–35.9	9.39–**18.7**–61.9	18.7–**21.0**–26.8[c]	14.7–**23.9**–32.3	12.6–**30.6**–65.0[g]
Coeff. Var. (%)[a]	49–**70**–126	90–**114**–180[b]	61–**77**–109[d]	93–**179**–237[cdg]	95–**129**–186[cg]
Count (sp/bur)[a]	60–**188**–223	53–**90** – 304	66–**111**–229	55–**233**–474[bdg]	62–**113**–194[bg]
Intensity (sp/s)[a]	2.24–**5.79**–15.5	3.80–**6.14**–8.84	3.89–**5.57**–8.88[c]	4.12–**9.78**–12.1[beg]	1.42–**3.43**–7.26[bdgh]
Period (s/c)[a]	62.1–**72.8**–108	57.3–**67.7**–105	42.5–**74.1**–110	52.5–**80.6**–96.2[g]	44.8–**56.8**–122[cd]
1000 ms criterion					
Duration (s)[a]	1.52–**3.59**–6.75	1.25–**1.73**–2.36[b]	1.07–**1.86**–3.26[b]	1.56–**4.11**–7.64[dg]	0.93–**3.23**–6.17[df]
Coeff. Var. (%)[a]	152–**218**–352	119–**145**–178[c]	150–**192**–257[d]	123–**169**–225[b]	119–**168**–218[c]
Count (sp/bur)[a]	13–**64**–158	18–**25**–35[c]	16–**21**–38[c]	15–**43**–132[eg]	13–**35**–132[cef]
Intensity (sp/s)[a]	6.70–**19.7**–29.7	11.9–**15.6**–31.6	10.8–**13.6**–18.9[b]	7.77–**16.1**–22.1[g]	4.32–**9.46**–17.3[beg]
Period (s/c)	14.8–**23.6**–48.3	6.86–**12.3**–24.9[b]	10.0–**17.6**–26.3	13.1–**17.5**–30.6[e]	13.8–**15.5**–65.6[d]
100 ms criterion					
Duration (ms)[a]	37–**115**–231	121–**134**–242[b]	42–**91**–146[d]	81–**99**–166[d]	76–**144**–232[gg]
Coeff. Var. (%)[a]	89–**140**–223	88–**99**–121[b]	89–**145**–217[d]	70–**93**–150[bfh]	89–**148**–221[d]
Count (sp/bur)[a]	4–**7**–19	7–**9**–19[b]	6–**8**–10[c]	5–**8**–11[b]	3–**7**–11[d]
Intensity (sp/s)[a]	75.3–**84.7**–119	49.3–**57.8**–64.9[c]	56.1–**81.2**–109[d]	28.2–**52.8**–83.0[cg]	24.2–**32.2**–66.7[cgd]
Period (ms/c)[a]	430–**500**–574	432–**461**–524	444–**535**–654	444–**502**–580[h]	381–**449**–484[cg]
10 ms criterion					
Duration (ms)[a]	10–**14**–18	10–**11**–15	9–**11**–16	10–**13**–17[fh]	5–**8**–11[cd]
Coeff. Var. (%)[a]	71–**83**–122	72–**81**–118	55–**76**–94[b]	76–**96**–115[fg]	53–**72**–94[bg]
Count (sp/bur)[a]	3–**4**–5	3–**4**–4	3–**3**–5	3–**4**–5[h]	2–**3**–4[bg]
Intensity (sp/s)	191–**222**–233	217–**227**–232	209–**220**–245	192–**206**–259[f]	186–**231**–370[g]
Period (ms/c)[a]	34–**43**–46	42–**44**–51	34–**40**–44	41–**43**–47[fh]	41–**49**–54[cg]

Abbreviations as in Table 1.

[a]overall *treatment* effect ($p < 0.05$); [b]$p < 0.05$, [c]$p < 0.01$ vis a vis the control cultures; [d]$p < 0.05$, [e]$p < 0.01$ vis a vis the bicuculline group; [f]$p < 0.05$, [g]$p < 0.01$ between the APV and the DNQX or APV + DNQX group; [h]$p < 0.05$, [i]$p < 0.01$ between the DNQX and the APV + DNQX group.

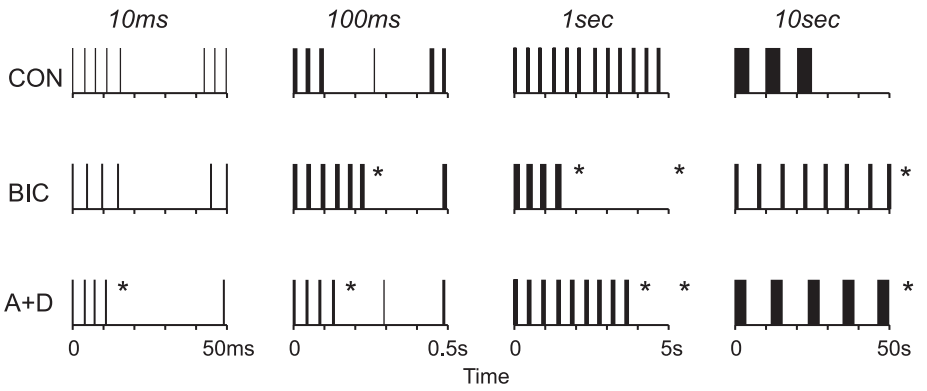

Fig. 4. Reconstruction of "modal firing patterns" in explants cultured for 2 weeks in the presence of different amino acid receptor blockers. Explanations as in Fig. 1.

Table 5. Spontaneous activity recorded in growth media containing selective amino acid receptor blockers: neuronal firing patterns after 3-weeks of culture in vitro

Group	Control (18)	Bicuculline (9)	APV + DNQX (16)
MFR (sp/sec)[b]	3.17–**4.99**–7.69	1.48–**3.77**–7.31	1.34–**2.73**–6.79[c]
Modal ISI (ms)a[b]	5–**8**–15	4–**5**–9	15–**35**–80[df]
Burst ratio[a]	0.90–**0.95**–0.98	0.94–**0.98**–0.98[e]	0.67–**0.80**–0.96[d]
CV-60 s (%)[ab]	78–**113**–173	45–**61**–113[c]	69–**75**–96[d]
10,000 ms criterion			
Duration (sec)[ab]	27.0–**32.2**–44.7	34.0–**50.2**–83.9[c]	56.0–**73.4**–143[d]
Coeff. Var. (%)[a]	56–**97**–119	86–**92**–248	106–**146**–159[d]
Count (sp/bur)[b]	221–**415**–781	95–**414**–607	203–**281**–825
Intensity (sp/s)[abb]	9.21–**12.2**–15.5	5.16–**8.56**–9.97	2.02–**3.40**–8.94[d]
Period (s/c)[ab]	67.7–**73.8**–96.3	62.8–**66.4**–128	75.0–**105**–200[d]
1000 ms criterion			
Duration (sec)[ab]	3.80–**4.82**–6.18	0.36–**1.41**–1.90[d]	1.88–**3.60**–6.10[e]
Coeff. Var. (%)[ab]	212–**238**–258	129–**171**–222[cd]	234–**280**–325[c]
Count (sp/bur)[ab]	53–**92**–113	19–**43**–48[c]	9–**22**–94[d]
Intensity (sp/s)[ab]	15.0–**17.4**–28.4	24.2–**37.4**–54.3[f]	5.36–**12.4**–21.6[d]
Period (s/c)[ab]	12.6–**14.3**–20.3	6.61–**7.50**–14.1[de]	7.89–**11.8**–25.3[d]
100 ms criterion			
Duration (ms)[a]	62–**97**–187	129–**224**–266[c]	89–**128**–202[e]
Coeff. Var. (%)[ab]	115–**226**–397	82–**95**–153[c]	76–**160**–416[e]
Count (sp/bur)[a]	8–**10**–13	9–**11**–14[e]	4–**6**–9[d]
Intensity (sp/s)[a]	50.6–**77.8**–114	47.6–**74.3**–105[e]	26.2–**35.2**–56.1[d]
Period (ms/c)	431–**442**–535	376–**478**–506	426–**482**–517
10 ms criterion			
Duration (ms)[a]	12–**15**–19	12–**13**–22	9–**11**–12[d]
Coeff. Var. (%)[b]	83–**112**–159	96–**98**–128	75–**89**–100[c]
Count (sp/bur)	4–**5**–6	4–**4**–6	3–**4**–4[d]
Intensity (sp/s)	200–**227**–268	219–**233**–241	194–**248**–281
Period (ms/c)	35–**42**–48	45–**46**–48[c]	37–**48**–50

Abbreviations as in Table 1.
[a]overall *treatment* effect ($p < 0.05$); [b]overall *age* effect (1–3 wiv: cf. tables 1 & 4; $p < 0.05$) [c]$p < 0.05$; [d]$p < 0.01$ vis a vis the control cultures; [e]$p < 0.05$, [f]$p < 0.01$ between the BICUC and the APV + DNQX group.

1000 ms criterion: *period*), but trains of such bursts lasted much longer before being interrupted by a period of quiescence.

Explants cultured in APV + DNQX, on the other hand, still suffered after 3 weeks from abnormally short micro-bursts (Fig. 4; Table 5: 10 ms criterion) the durations of which, as in the bicuculline group, were more consistent throughout a given recording session than in control explants. Network bursts in this group were somewhat shorter and even more variable than in the controls; the intensity of firing during such bursts was reduced as well, which was partially compensated for by the shorter intervals between them (Table 5: 1000 ms criterion). The most striking effect of chronic APV + DNQX treatment was the extreme, albeit highly variable, prolongation of (network-)burst trains in the combined presence of these two glutamate receptor blocking agents (also see Fig. 4).

The even stronger "cocktail" of glutamate receptor blockers (LAD group) caused a still more drastic reduction in micro-bursting (Table 6: 10 ms criterion), along with profound lengthening of modal interspike intervals (all in all, very similar to the situation observed at earlier ages: see Fig. 2). Perhaps not unexpectedly under these conditions, an unusually high proportion of spontaneous action potentials was scored as "background" activity, c.q. isolated spikes (see Table 6: *burst ratio*).This strong reduction in neuronal firing at the micro-burst level

Table 6. Spontaneous firing patterns at 3-weeks in vitro during chronic blockade of AMPA, NMDA and kainate receptors (LAD group)

	Control (16)	LAD (21)
MFR (sp/s)[c]	2.50–**4.98**–8.48	1.29–**3.75**–5.71[ad]
Modal ISI(ms)[c]	2–**4**–7	16–**30**–73[b]
Burst ratio[c]	0.97–**0.98**–0.99	0.62–**0.82**–0.88[b]
CV-60 s (%)[c]	52–**90**–131	49–**86**–126
1000 ms criterion		
Duration (s)[c]	1.43–**3.94**–6.14	1.44–**3.58**–4.75
Coeff.Var. (%)[c]	113–**155**–181	126–**170**–223[a]
Count (sp/bur)[c]	54–**125**–179	15–**42**–77[b]
Intensity (sp/s)[c]	29.9–**36.6**–44.9	4.48–**9.83**–14.6[b]
Period (s/c)[c]	7.64–**18.7**–333	3 8.85–**12.0**–16.6[a]
100 ms criterion		
Duration (ms)[c]	144–**190**–294	101–**191**–268
Coeff. Var. (%)[c]	119–**166**–256	91–**18**–286
Count (sp/bur)[c]	12–**18**–24	3–**8**–11
Intensity (sp/s)	73.7–**88.0**–103	21.3–**29.0**–43.2
Period (ms/c)	398–**441**–459	404–**456**–477
10 ms criterion		
Duration (ms)	11–**13**–21	6–**8**–10
Coeff. Var. (%)[c]	92–**98**–109	46–**74**–106
Count (sp/bur)	4–**5**–7	2–**2**–3
Intensity (sp/s)	234–**259**–306	181–**190**–246
Period (ms/c)[a]	42–**47**–51	42–**45**–51

Abbreviations as in Table 1. [a]$p < 0.05$, [b]$p < 0.01$ vis a vis the control group. [c]$p < 0.05$ for age differences among the LAD groups (cf. Table 3). [d] includes 3 silent cases which later became active in control medium.

manifested itself on the higher time-scales as well, although it was partly compensated for by an enhanced incidence of network bursts (see Fig. 2).

Pharmacological dissection of putative mechanisms underlying chronically induced functional deficits

Some of the 2-week-old cultures were acutely exposed to selective synaptic receptor blockers in order to get some idea of where changes at the cellular level underlying the observed abnormalities in network activity patterns might be localized. In contrast to 1-week-old cultures, NMDA blockade alone failed to have much effect on overall activity levels in control cultures, but notably decreased the *duration* of burst discharges without, however, affecting their *intensity* of firing (Table 7). AMPA receptor blockade in addition (i.e., the acute APV + DNQX group) led to the virtual disappearance of spontaneous firing in all cases. Such activity as remained took the form of abnormally long, very low intensity network bursts at long intervals, with a high proportion of isolated spikes in between (see Table 7: 1000 ms criterion and burst ratio). Mini-bursts, too, were greatly weakened by acute APV + DNQX treatment, although they continued to occur in short trains with an impressively regular periodicity (see Table 7: 100 ms criterion). This residual activity could be eliminated by selectively blocking the kainate receptors with LY (data not shown).

Combined blockade of AMPA and NMDA receptors was also carried out by acutely adding the complementary blocking agent to cultures which had been treated chronically with either APV or DNQX. In this way the effects of *selective* chronic glutamate receptor blockade could be assayed under identical conditions in both cases (Table 8). Neither of these chronically treated groups showed anything like the reduction in spontaneous activity caused by acute

Table 7. Spontaneous firing patterns at 2-weeks in vitro in control cultures following **acute** blockade of AMPA and/or NMDA receptors: treatment with APV + DNQX or with APV-only

Group	APV-only (8)	APV + DNQX (4)
MFR (sp/s)[d]	1.14–**1.92**–2.42	0.24–**0.30**–0.36[b]
Burst Ratio[d]	0.85–**0.92**–0.94	0.37–**0.39**–0.40[c]
CV-60s (%)	42–**61**–67[c]	148–**161**–183[c]
1000 ms criterion		
Duration (s)[c]	0.26–**0.41**–1.14[b]	1.61–**1.84**–2.06
Coeff. Var. (%)[d]	129–**186**–339[c]	126–**144**–161
Count (sp/bur)	8–**10**–12[c]	7–**8**–8[b]
Intensity (sp/s)[d]	17.1–**26.4**–30.4	5.18–**5.26**–5.34[b]
Period (s/c)[d]	3.16–**4.63**–4.74[b]	24.3–**30.9**–37.4
100 ms criterion		
Duration (ms)[c]	51–**57**–62[b]	75–**83**–91[c]
Coeff. Var. (%)[c]	85–**99**–164	74–**77**–79[c]
Count (sp/bur)[c]	6–**7**–7[c]	3–**3**–3[c]
Intensity (sp/s)[d]	92.7–**95.7**–138[c]	23.0–**25.3**–27.7[c]
Period (ms/c)	450–**488**–531	416–**432**–447

Abbreviations as in Table 1.
[c]$p < 0.05$, [b]$p < 0.01$ vis a vis the corresponding *chronically* treated group (see Table 4); [c]$p < 0.05$, [d]$p < 0.01$ with respect to each other.

Table 8. Acute effects of DNQX or APV added to the growth medium in 2-week-old cultures grown continuously in the presence of, respectively, APV ($n = 18$) or DNQX ($n = 12$)

Group	chronic APV (+DNQX)	chronic DNQX (+APV)
MFR (sp/s)[d]	0.37–**0.84**–1.15	1.06–**1.47**–1.83[b]
Burst Ratio[c]	0.88–**0.94**–0.98	0.98–**0.99**–1.00[b]
CV-60 s (%)[d]	108–**155**–253[b]	80–**93**–135
1000 ms criterion		
Duration (s)	0.69–**1.25**–2.25[b]	0.68–**1.24**–1.87[b]
Coeff. Var. (%)	147–**183**–225	169–**208**–227
Count (sp/bur)[a]	10–**13**–24[b]	15–**23**–42[b]
Intensity (sp/s)	11.7–**15.2**–21.9[a]	14.3–**17.4**–39.5
Period (s/c)[c]	8.70–**10.9**–20.1	12.2–**19.3**–24.1
100 ms criterion		
Duration (ms)[a]	38–**85**–112[b]	80–**131**–172[a]
Coeff. Var. (%)	85–**120**–156[b]	75–**97**–150[b]
Count (sp/bur)[d]	5–**6**–7	7–**9**–12
Intensity (sp/s)	53.9–**77.0**–139[b]	42.7–**86.1**–112[b]
Period (ms/c)	420–**464**–628	391–**497**–647

Abbreviations as in Table 1.
[a]$p < 0.05$, [b]$p < 0.01$ vis a vis the *chronic* APV + DNQX group (see Table 4); [c]$p < 0.05$, [d]$p < 0.01$ vis a vis each other.

treatment. Here too, residual firing was sensitive to LY treatment, so that a considerable degree of compensation by way of increased kainate receptor mediated synaptic drive must be presumed. The two groups were fairly similar in all respects but with significantly greater recovery of normal function in the chronic DNQX than in the APV group; indeed, the former were virtually indistinguishable from control values (cf. Tables 4 and 8).

The logical conclusion would seem to be that kainate receptors, which normally play only a minor role in sustaining spontaneous firing (see above),

Table 9. Spontaneous firing patterns recorded in the growth medium (R16) at 2-weeks in vitro in normal control ($n = 9$) vs. a parallel group of chronically DNQX-treated cultures ($n = 12$) under conditions of AMPA receptor blockade (acute DNQX application) before and immediately after the addition of the GABAergic receptor blocker bicuculline

	Acute Dnqx		(“) + Bicuculline	
Group	Control	Chronic DNQX	Control	Chronic DNQX
MFR (sp/s)	0.20–**0.47**–0.91[f]	2.02–**3.90**–5.42[b]	0.27–**0.56**–2.41[df]	1.27–**2.33**–2.69[a]
Burst Ratio	0.44–**0.69**–0.85[f]	0.72–**0.94**–0.98[a]	0.80–**0.86**–0.92[e]	0.86–**0.90**–0.93
CV-60s (%)	176–**197**–244[f]	68–**83**–105[b]	146–**219**–245[df]	45–**65**–84[b]
1000 ms criterion				
Duration (s)	1.27–**1.42**–2.17[e]	2.00–**3.31**–4.88[b]	1.39–**2.47**–2.69[ce]	1.24–**1.41**–2.63
Coeff. Var. (%)	121–**147**–163[e]	150–**204**–227[a]	133–**159**–197[ce]	82–**133**–201
Count (sp/bur)	10–**12**–35[f]	24–**60**–94[a]	23–**43**–45[ce]	18–**23**–36
Intensity (sp/s)	6.50–**16.5**–18.9	7.78–**17.0**–19.5	12.8–**16.6**–22.0	14.1–**17.8**–22.7
Period (s/c)	51.3–**59.0**–64.7[f]	12.7–**15.9**–26.7[b]	12.9–**35.9**–83.3[c]	8.11–**8.91**–11.7[bc]
100 ms criterion				
Duration (ms)	81–**90**–231	89–**133**–147	129–**271**–489[ce]	84–**110**–177[a]
Coeff. Var. (%)	77–**102**–156	70–**88**–150	122–**177**–226[c]	68–**100**–159
Count (sp/bur)	3–**8**–17	5–**10**–11	7–**9**–33	7–**9**–13[a]
Intensity (sp/s)	33.8–**51.0**–71.3[f]	37.0–**56.6**–75.8	39.4–**56.4**–73.5[f]	62.9–**74.5**–86.4
Period (ms/c)	410–**436**–538	444–**472**–574	396–**417**–464[ce]	423–**430**–444

Abbreviations as in Table 1.
[a]$p < 0.05$; [b]$p < 0.01$ vis a vis the corresponding control group; [c]$p < 0.05$; [d]$p < 0.01$ vis a vis the chronic-DNQX group without added bicuculline; [e]$p < 0.05$, [f]$p < 0.01$ vis a vis 2-week-old controls in normal growth medium (see Table 4). [All groups assayed in the presence of DNQX.]

become effectively upregulated to a greater degree when AMPA rather than NMDA receptors are chronically blocked, even to the point of fully compensating for the absence of the usual major sources of synaptic drive. In order to check the possibility that *down*regulation of inhibitory synapses may have contributed to this functional recovery, the effect of bicuculline was studied in control cultures in which AMPA receptors were acutely blocked by DNQX, and compared with the bicuculline effect in a chronic DNQX-treated group recorded while their AMPA receptors were still blocked. In contrast to the enormously enhanced and relatively steady firing observed in the chronically DNQX-treated explants (Table 9), acutely disinhibited control explants showed merely an increase in stereotyped bursting without any increase in overall mean firing levels nor reduction in minute-to-minute fluctuations. When the chronic-DNQX cultures, too, were acutely treated with bicuculline they showed the expected increase in network bursting, but with the persistence of very large differences in overall activity from the corresponding control group (Fig. 9). This hyperactivity, therefore, must be attributed to an upregulation of excitatory mechanisms rather than to a weakening of synaptic inhibition.

Experiments have recently been initiated with the aim of pinpointing the sources of excitatory drive in the absence of functioning glutamate receptors. Since the presence of cholinergic interneurons has been reported, spread throughout the neocortex, and since most of the cholinergic neurons in the brain are muscarinic (see Nieuwenhuys, 1985), atropine was added acutely to the growth medium of chronically LAD-treated explants in the expectation that spontaneous discharges might then disappear. Ongoing network burst activity was indeed suppressed but, to our amazement, overall firing levels became considerably enhanced as a result of sustained “tonic” firing throughout the remainder of the recording session (Fig. 5). This novel activity pattern showed characteristically long interspike intervals on the whole (Table 10: *modal ISI*), but also contained short trains of “mini-bursts” at unusually regular intervals of ca. 450 ms, during which background spiking was frequently seen (Table 10: 100 ms criterion). Each

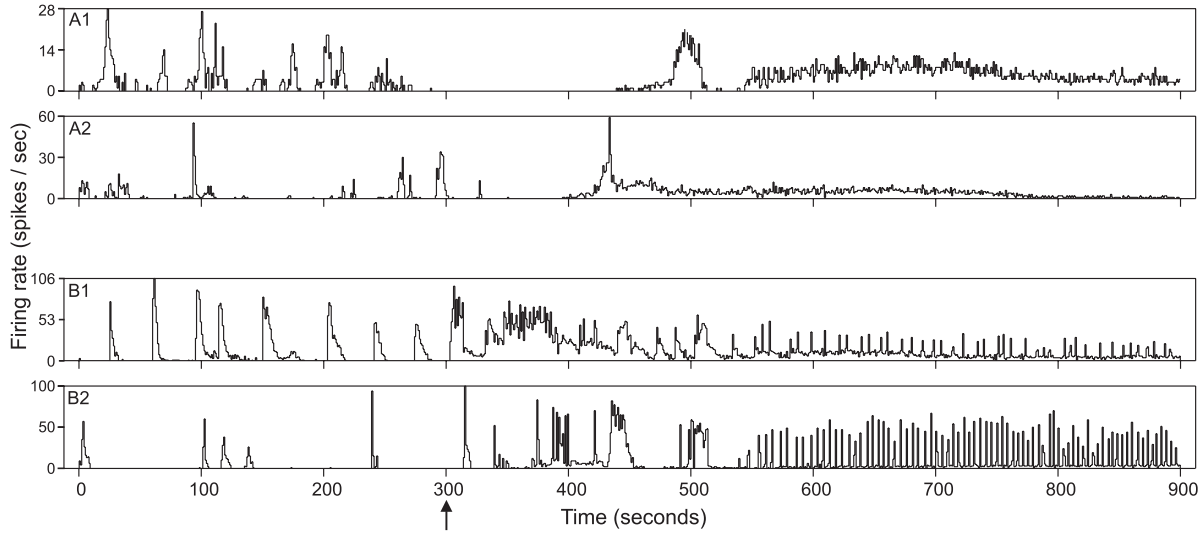

Fig. 5. Acute effect of atropine administration (at 300 s: arrow) in two chronic LAD-treated 2-week-old cultures (A1, 2), showing the characteristic rapid appearance of sustained "background" firing and suppression of repetitive bursting (but note the quantitative differences between preparations). Also in control cultures (B1, 2), trains of bursts are suppressed in favor of sustained low-level "tonic" firing; in this case, however, brief bursts continue to occur (but at much more regular intervals) rather than being grouped into trains, separated by long intervals of relative quiescence. Firing rates are presented as the number of spikes in consecutive 1-s bins over a 15-min period.

Table 10. Spontaneous firing patterns elicited by addition of atropine to the growth medium in 2-week-old explants cultured either with or without glutamate receptor blockade (LAD group)

	Control (6)	LAD (12)
MFR (sp/s)	4.66–**9.26**–14.3	2.86–**4.56**–6.38[a]
Modal ISI (ms)	2–**2**–3	113–**132**–200[a]
100 ms criterion		
Duration (ms)	136–**157**–211	93–**106**–155[a]
Burst Ratio (%)	0.91–0.**96**–0.99	0.68–**0.74**–0.76[a]
Intensity (sp/s)	77.3–**82.5**–103	23.2–**27.4**–32.9[a]
Period (ms/c)	324–**379**–426	443–**452**–468[a]
Coeff. Var. (%)	56–**70**–72	45–**46**–47
10 ms criterion		
Duration (ms)	14–**19**–28	6–**8**–9[a]
Burst Ratio (%)	0.78–0.**84**–0.86	0.12–**0.21**–0.30[a]
Intensity (sp/s)	327–**331**–335	176–**182**–198[a]
Period (ms/c)	51–**54**–56	49–**50**–51[a]
Coeff. Var. (%)	38–**41**–44	41–**44**–48

Abbreviations as in Table 1.
[a] $p < 0.05$ for the difference from the control group.

mini-burst, in turn, was made up of ca. three very short micro-bursts (usually merely spike "doublets") occurring with great regularity every ca. 50 ms and separated by an ongoing background of isolated action potentials (Table 10: 10 ms criterion). Addition of a selective *nicotinic* receptor blocker (dihydro-β-erythroidine hydrobromide: Sigma) failed to noticeably influence these ongoing SBA patterns.

The inescapable conclusion seems to be that cholinergic mechanisms are only partly responsible for the homeostatic physiological response to long-lasting total glutamate receptor blockade but that they also exert an unexpected inhibitory action on the network's tendency toward tonic low-level firing in between bursts. Since acute LAD administration completely suppresses extracellularly recorded SBA at all ages studied (data not shown), there appears to have been an upregulation of excitatory muscarinic receptors under these conditions. The question nevertheless arises of a possible contribution of cholinergic neurons to spontaneous firing patterns even in *control* cortical explants. In a series of experiments designed to address this question, an acute disinhibitory effect of atropine on background activity was indeed consistently seen in this group as well, with the induced tonic firing tending to obscure the continued presence of repetitive ''network bursts'', albeit at unusually short and regular intervals (see Fig. 5; Table 9). Undoubtedly due to the presence of functioning glutamatergic synapses in the control cultures, moreover, both mini- and micro-burst durations were considerably longer, and the firing more intense than in chronic LAD-treated cultures, although the mean intervals between successive bursts hardly differed between the two groups (Table 10: *period*).

Theoretical evaluation of compensatory neurophysiological mechanisms during neocortical network formation

Plasticity of glutamatergic mechanisms

The most striking of our recent findings is the enormous flexibility of developing occipital cortical co-cultures to disturbances in their intrinsic neuronal firing. It was already known from experiments involving isolated cultures that selective NMDA receptor blockade can induce upregulation of AMPA receptors to the point that normal activity levels are attained despite the blockade, and with strongly intensified firing when assayed in control medium (Corner et al., 2002). In contrast, selective AMPA receptor blockade in isolated explants slowly depressed the networks' spontaneous activity, whereas combined pharmacological blockade of both receptor types eliminated it altogether for as long as treatment was continued. In the co-cultures described in the present report, on the other hand, AMPA receptor blockade failed to prevent SBA from soon returning almost to its normal level even when NMDA receptors were simultaneously blocked. With both of these treatments, SBA stayed at control levels for at least 1–2 weeks in vitro. Subsequent acute selective blockade of kainate receptors, while still in the growth medium, then revealed that this class of glutamate receptors (which normally facilitates SBA but is incapable of generating it on its own) had upregulated so as to largely compensate for the absence of the other two synaptic receptor classes. A major structural difference between isolated explants and explants co-cultured in a manner permitting mutual innervation is the extent of dendritic arborization, which occurs normally in the latter group but is arrested in the former (Baker and van Pelt, 1997). Either the increased membrane surface provided by fully branched dendritic trees or a secondary effect of the putative trophic factor responsible for the enhanced outgrowth in co-cultures could, in principle, be involved in the extraordinary plasticity demonstrated in the present experiments.

Plasticity in cholinergic systems

Even when all three glutamate receptor types were chronically blocked throughout the period of functional network formation, SBA was found to have become partially restored within a few days, and gradually approached normal levels over the next two weeks. The recurrent burst discharges which characterized this, as well as the firing patterns observed in the other groups at all ages, were eliminated by selective blockade of muscarinic acetylcholine receptors, an effect not observed in control cultures. Not only must this group of receptors, therefore, have upregulated (Baker et al., 1992) to the point of being able to sustain some degree of ongoing firing on their own, but the presence of functioning cholinergic neurons in these explants must also be presumed. The fact that their presence in the network was not in itself a consequence of the suppression of SBA could be demonstrated by also subjecting some of the

control cultures to treatment with atropine. The effect was completely unexpected but consistent, viz., the appearance of sustained "background" firing throughout the intervals between successive bursts. Since this effect of atropine was also observed unequivocally in the experimental group, a tonic cholinergic inhibition of the tendency for individual neurons to fire tonically in between bursts of synchronized network activity must be postulated in both cases. The presence of acetylcholine containing neurons scattered throughout the neocortex was already reported in intact rat brain (Nieuwenhuys, 1985) so that one need not suppose that their presence in vitro is an "artifact" of culture conditions. The possibility that the unmasked tonic excitatory drive is itself cholinergic, but mediated via nicotinic rather than muscarinic receptors, seems highly unlikely (see above).

It was equally surprising to find that the "fine structure" of SBA was preserved even when the spike trains were transformed by atropine from phasic to tonic firing: the observed periodicities at "mini-" and "micro-" time-scales (viz., 2–3 Hz. and ca. 25 Hz., respectively) remained unchanged despite the qualitative transition in the firing pattern. It would be premature to speculate here about ion-channel kinetics and other putative sources of rhythmic patterning of spike discharges (Steriade et al., 1990), but it is clear that great differences in "plasticity" in the face of altered physiological conditions must exist at the cellular and membrane levels. Thus, whereas most of the parameters describing the relatively long "network" bursts, as well as their clustering into alternating epochs of high and low activity, were strongly and differentially affected by the various functional challenges employed in the present experiments, on faster time-scales there was hardly any effect no matter what treatment was employed. Thus periodicities of, on average, ca. 45 and 450 ms. were observed with high reproducibility from preparation to preparation at all ages and under all experimental conditions. Besides the persistence of these "fast rhythms" even during periods of tonic firing, they showed extreme resistance to change also when bursting had been intensified either acutely or chronically by bicuculline mediated disinhibition from GABAergic synaptic drive. In this respect there is a welcome similarity to the behavior of the neocortex in vivo which, when neuronally isolated, spontaneously generates similar rhythms in its field potentials (EEG "*beta*" and "*delta*" frequencies: Gastaut) and concomitant spike trains, and which are prominently present in the intact cortical SBA whether the animal is asleep or aroused (thus associated with, respectively, "synchronized" or "desynchronized" EEG patterns: Bullock and Basar, 1988).

Activity-dependent "homeostatic" mechanisms

The prevalence of spontaneous activity and the tenaciousness whereby developing cortical networks "homeostatically" regulate it in the direction of normality when confronted with functional imbalances (see Corner and Ramakers, 1992; Davis and Bezprozvanny, 2001; Corner et al., 2002, 2004; Burrone and Murthy, 2003) implicates such activity as being important for the development of the mature balance among excitatory and inhibitory neuronal mechanisms. A similar line of argument was already pursued many years ago with respect to the ontogenetic significance of REM sleep (Roffwarg et al., 1966), and was proven by later experiments to have been a very fruitful suggestion indeed (for review, see Mirmiran, 1995). Experimental support for a developmental role of SBA in cortical development in vitro, too, is now amply available for several simplified preparations, and will be shown in a forthcoming paper to hold true also for the more realistic "model" system, viz. cross-innervated organotypic co-cultures, which form the basis of the present report (Corner et al., 2005). Thus, while neuronal firing following recovery from tetrodotoxin induced total suppression of SBA perfectly well mimics the epileptiform discharges previously reported for isolated explants and dissociated cell cultures (reviewed in Corner et al., 2002), paired explants subjected to partial SBA blockade (i.e., the present experiments) will be shown to display relatively minor abnormalities, commensurate with the high degree of restoration of normal firing levels during the period of network formation (Corner et al., 2004).

It would thus appear that, while homeostatic mechanisms may not always succeed in perfectly restoring normal spontaneous activity patterns, they

can go a long way toward protecting the network against the devastating hyperexcitability which would otherwise ensue when critical excitatory mechanisms are severely compromised at sensitive points in development (Corner and Ramakers, 1992; Corner et al., 2002, 2004). The precise onset and extent of such ''critical'' periods, and the degree to which damage to a neural network can be reversed by subsequent ''therapeutic'' stimulation or other treatment, is an important question which deserves to be systematically addressed now that the developing brain's vulnerability to functional disturbances has become well established.

It seems curious in regard of the foregoing considerations that there is such a large degree of individual variation within all types of preparations. The question rises, why does not *each individual explant* up- or downregulate its activity toward whatever the appropriate level and pattern for optimal maturation might be, thus giving negligible variance for all relevant parameters? One possibility is that the ''set-points'' for such regulation (the origins of which are poorly understood) are heterogeneously distributed such that even in the normal intact cerebral cortex there are patches of tissue with widely differing intrinsic excitabilities. Alternatively, set-points might become differentiated after transfer to in vitro conditions, based upon differences among the explants arising in the course of preparation.

An intriguing third possibility, however, is that the high SBA variances are not reflective of differential homeo static tendencies at all but, on the contrary, reflect varying levels of activity required to maintain all of the networks at a common preset excitability level, in ''forward reference'' to the evolutionarily determined need for optimal responsiveness to incoming stimuli. Thus, inhibitory interneurons (which constitute only a small minority of cortical neurons: Luhmann and Prince, 1991) could become unevenly distributed among the explants prepared for culture, by virtue of differences in the amount of damage, exact size or place of origin, leading in turn to differences in the degree to which the network's inherent tendency toward paroxysmal discharges are able to be counteracted. Even in the absence of surgical ''jitter'' during preparation, furthermore, probabilistic rules for establishing effective synaptic connections can in themselves lead to large regional differences in the functional integration of the inhibitory neurons, with consequent proportional differences in emerging spontaneous activity levels in different parts of the system (Van Ooyen and Van Pelt, 1996). When very small regions are isolated, such differences could lead to large functional variations which would be averaged out only in sufficiently large networks.

In this view, then, SBA would constitute a built-in monitor of developing network excitability, being high when inhibition is relatively sparse or weak, and low when inhibitory drive is initially too strong. The activity dependence of inhibitory neuronal maturation (reviewed in Corner et al., 2002; also Meier et al., 2003) would then provide a straighforward means for such initial imbalances to be gradually corrected as development proceeds. The same line of reasoning can be applied to the now well documented activity dependence of immature *excitatory* neurons, which operates in the opposite directions so as to correct the network's initial tendency toward excessive spine synapse formation and hypersensitivity to NMDA mediated excitation (see Baker et al., 1992; Van Ooyen and Van Pelt, 1996; Corner et al., 2002). In other words, a variety of mechanisms appear to work synergistically, using intrinsically generated bioelectric activity as a monitoring signal, to homeostatically regulate the development of cortical network excitability. Since repetitive generalized burst firing is a well-nigh universal developmental phenomenon in the central nervous system (also see Crain, 1976; Ben-Ari, 2001), it would not be surprising to find that its importance for neuroplasticity is not confined to the cerebral cortex. Indeed, it has been implicated even in such a presumably rigid structure as the spinal cord (reviewed in Corner, 1994).

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