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Recovery of network-driven glutamatergic activity in rat hippocampal neurons during chronic glutamate receptor blockade

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Abstract

Previous studies indicated that a long-term decrease in the activity of ionotropic glutamate receptors induces cholinergic activity in rat and mouse hypothalamic neuronal cultures. Here we studied whether a prolonged inactivation of ionotropic glutamate receptors also induces cholinergic activity in hippocampal neurons. Receptor activity was chronically suppressed in rat hippocampal primary neuronal cultures with two proportionally increasing sets of concentrations of NMDA plus non-NMDA receptor antagonists: 100 μ M/10 μ M AP5/CNQX (*1X cultures*) and 200 μ M/20 μ M AP5/CNQX (*2X cultures*). Using calcium imaging we demonstrate that cholinergic activity does not develop in these cultures. Instead, network-driven glutamate-dependent activity, that normally is detected in hyper-excitable conditions, reappears in each culture group in the presence of these antagonists and can be reversibly suppressed by higher concentrations of AP5/CNQX. This activity is mediated by non-NMDA receptors and is modulated by NMDA receptors. Further, non-NMDA receptors, the general level of glutamate receptor activity and CaMK-dependent signaling are critical for development of this network-driven glutamatergic activity in the presence of receptor antagonists. Using electrophysiology, western blotting and calcium imaging we show that some neuronal parameters are either reduced or not affected by chronic glutamate receptor blockade. However, other parameters (including neuronal excitability, mEPSC frequency, and expression of GluR1, NR1 and β CaMKII) become up-regulated and, in some cases, proportionally between the non-treated, 1X and 2X cultures. Our data suggest recovery of the network-driven glutamatergic activity after chronic glutamate receptor blockade. This recovery may represent a form of neuronal plasticity that compensates for the prolonged suppression of the activity of glutamate receptors.

Keywords

non-NMDA receptors; NMDA receptors; plasticity; CaMKII; acetylcholine

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1. Introduction

Specification of neurotransmitter phenotype is critical for neural circuit development and is influenced by intrinsic and extrinsic factors, including calcium signaling, cascades of transcription factors and target-derived neurotrophic factors (Goridis and Rohrer, 2002; Landis, 1996; Spitzer et al., 2004). Our previous findings in the rat and mouse hypothalamus *in vitro* and *in vivo* suggested the role of neurotransmitter glutamate in the regulation of cholinergic phenotype in neurons (Belousov et al., 2002; Belousov et al., 2001; Liu et al., 2008). The studies demonstrated that a prolonged (2–3 week) inactivation of ionotropic glutamate receptors increases the number of cholinergic neurons, the expression of acetylcholine (ACh) receptors, and induces ACh-dependent excitatory synaptic activity (Belousov et al., 2002; Belousov et al., 2001). The data suggested the importance of *N*-methyl-D-aspartate (NMDA) receptors and Ca^{2+} -dependent signaling in the cholinergic up-regulation and indicated that cholinergic phenotypic properties are induced in glutamate-secreting neurons (Belousov et al., 2002; Liu et al., 2008). In addition, the data implied that the mechanisms for cholinergic regulation have region-specific character. For example, the blockade of ionotropic glutamate receptors induces ACh-dependent excitatory activity in neuronal cultures prepared from the hypothalamus and cerebellum, but not from the neocortex (Belousov et al., 2002; Belousov et al., 2001). We speculated that glutamate-dependent regulation of cholinergic properties contributes to the neurotransmitter phenotype specification in developing neurons (Liu et al., 2008) and also represents a form of compensatory regulation that is expressed in both developing and mature neurons during the prolonged decrease in glutamate synaptic transmission (Belousov et al., 2001).

Here we extended our studies to determine whether or not a prolonged inactivation of ionotropic glutamate receptors also induces ACh-dependent excitatory activity in hippocampal neuronal cultures and, if not, whether other forms of compensatory regulation become expressed. We found that cholinergic activity does not develop in these cultures. Instead, network-driven glutamate-dependent activity, that normally is detected in hyper-excitable conditions, reappears in neurons in the presence of glutamate receptor antagonists. Given that inactivation of glutamate receptors induces homeostatic changes in glutamate-dependent activity in neuronal networks (Thiagarajan et al., 2005; Thiagarajan et al., 2002; Turrigiano, 2007; Turrigiano et al., 1998) and glutamate receptor antagonists are considered for the use in clinical conditions, we studied the dynamics of reappearance of glutamatergic activity during prolonged inactivation of glutamate receptors and the mechanisms responsible for this reappearance.

2. Results

2.1. Does ACh-dependent activity develop in hippocampal neurons?

Previous calcium imaging and electrophysiological studies revealed the development of ACh-dependent excitatory postsynaptic activity in hypothalamic neuronal cultures after chronic blockade of ionotropic glutamate receptors with an NMDA and non-NMDA receptor antagonists D,L-2-amino-5-phosphonovalerate (AP5; 100 μM) plus 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) (*1X AP5/CNQX concentrations*) (Belousov et al., 2004; Belousov et al., 2001). In those studies, the cholinergic activity was evident in many neurons (55–70%) during acute neuronal disinhibition with the γ -aminobutyric acid A receptor ($\text{GABA}_\text{A}\text{R}$) antagonist, bicuculline (applied in the continued presence of *1X AP5/CNQX*), and was suppressed by nicotinic and muscarinic ACh receptor antagonists. To determine whether ACh-dependent activity also develops in hippocampal neurons, Ca^{2+} imaging tests were conducted in three groups of hippocampal neuronal cultures: non-treated (*control*) cultures; non-treated cultures in which 100/10 μM AP5/CNQX were added acutely for 30 min (*acute AP5/CNQX cultures*); and cultures that were chronically treated for 3–6 weeks, starting day

in vitro 4 (DIV4) with 100/10 μM AP5/CNQX (1X cultures) (see Experimental procedures). All cultures were tested 3.5–6.5 weeks after the culture preparation. In control cultures, all neurons ($n=62$ of 62; 100%) exhibited the high-frequency large intracellular Ca^{2+} oscillations during the application of bicuculline (50 μM ; Fig. 1A and Table 1). The oscillations were glutamatergic in nature as they were quickly and completely suppressed in these neurons by an administration of 1X AP5/CNQX ($n=62$; Fig. 1A and Table 1) and were never detected in other neurons ($n=477$) that were specifically tested for the presence of bicuculline-mediated intracellular Ca^{2+} oscillations in acute AP5/CNQX culture conditions (Fig. 1B and Table 1). In contrast in 1X cultures, an application of bicuculline induced large intracellular Ca^{2+} oscillations in many neurons ($n=724$ of 1201; 60.3%) even in the presence of 100/10 μM AP5/CNQX (Fig. 1C and Table 1). However, compared to 1X hypothalamic cultures, where most of such the bicuculline-mediated activity is cholinergic (Belousov et al., 2004; Belousov et al., 2001), the bicuculline-mediated activity in 1X hippocampal cultures was not affected by ACh receptor antagonists, atropine plus mecamylamine (100 μM each; $n=213$ tested; Fig. 1C, 2F), suggesting that it is not of a cholinergic origin. Thus, the data indicate that although some bicuculline-mediated oscillatory Ca^{2+} activity appears in hippocampal neuronal cultures after chronic glutamate receptor blockade, ACh-dependent activity does not develop in these cultures.

2.2. Occurrence and nature of the bicuculline-mediated activity in hippocampal neurons

We set out to characterize the occurrence and to determine the nature of bicuculline-mediated oscillatory Ca^{2+} activity that is detected in the presence of glutamate receptor antagonists in 1X hippocampal cultures. In cultures that were chronically treated with 1X AP5/CNQX for 3–6 weeks, the incidence of neuronal responsiveness to bicuculline was generally dependent on individual culturing events: both more-responsive and less-responsive batches were found (see Experimental procedures/4.4. Ca^{2+} imaging). The expression of bicuculline-mediated activity also was field-specific. On average, 20–40 neurons were tested in each microscope field. If bicuculline-mediated activity was detected in neurons in a microscope field, all (or nearly all) neurons showed such the activity ($n=26$ of 43 fields; Table 1). In bicuculline-non-responsive fields ($n=17$ of 43), none of the neurons demonstrated the bicuculline-mediated Ca^{2+} oscillations. In fields that were bicuculline-responsive, the activity was synchronized between all neurons in the same field in Ca^{2+} imaging recordings (Fig. 2A) and appeared as synchronized giant EPSPs in dual electrophysiological recordings ($n=6$ neuronal pairs; Fig. 2B). Further, in young cultures the bicuculline-mediated activity appeared after >10 days of 1X treatment - it was not yet detected on DIV11–14 when the treatment started on DIV4 (Fig. 2C). However, the activity appeared much faster in mature cultures - it was seen in DIV34 neurons after only 4 days of 1X treatment (i.e., when the treatment started on DIV30; Fig. 2D).

We did additional experiments in bicuculline-non-responsive fields in 1X cultures to see whether or not the bicuculline-mediated Ca^{2+} oscillations can appear at the reduced concentrations of glutamate receptor antagonists. In these fields, indeed, the bicuculline-mediated Ca^{2+} oscillations were detected when the concentrations of AP5/CNQX were lowered to $0.48 \pm 0.07\text{X}$ ($n=5$ fields, 107 neurons; not shown). In fact, all these fields demonstrated Ca^{2+} activity at 0.3X. In the meantime, in control cultures, no bicuculline-mediated oscillations were expressed at 0.3X ($n=5$ fields, 110 neurons; not shown) and appeared only in the absence of AP5/CNQX.

To determine nature of the bicuculline-mediated activity that appears in hippocampal neurons after chronic 1X treatment, tests were done in bicuculline-responsive fields using the pharmacological inactivation of neurotransmitter receptors and gap junctions. In 1X cultures (treated on DIV4–30, tested on DIV30), the bicuculline-mediated Ca^{2+} oscillations were not affected by the blockade of gap junctions (with carbenoxolone, 20 μM ; Fig. 3F) or by the

doubled concentration of AP5 (200 μ M total; Fig. 3A, F), but were increased after AP5 removal, i.e., in the presence of CNQX only (Fig. 3B, F). However, the oscillations were completely eliminated by the doubled concentration of AP5/CNQX (200/20 μ M total; Fig. 3C, F), by the doubled concentration of CNQX (20 μ M total; Fig. 3D, F), by 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μ M; another non-NMDA glutamate receptor antagonist; Fig. 3F), or tetrodotoxin (TTX, 2 μ M; voltage-gated sodium channel blocker; Fig. 3F), and were dramatically increased (along with the increase in background Ca^{2+} level) after CNQX removal, i.e., in the presence of AP5 only (Fig. 3E, F).

Thus, altogether, the data suggest that the network-driven (i.e., disinhibition-mediated) glutamatergic activity is expressed in mature control hippocampal neuronal cultures and is suppressed by acute administration of glutamate receptor antagonists. However, this activity appears in the presence of these antagonists if the neurons are treated with the antagonists chronically. Further, in 1X cultures this activity is mediated by non-NMDA receptors and is modulated by NMDA receptors. Given that the network-driven glutamatergic activity also is expressed in non-treated developing hippocampal neurons on DIV10 and is reversibly suppressed by ionotropic glutamate receptor antagonists (Arnold et al., 2005), our data suggest the recovery of this activity after chronic glutamate receptor blockade.

2.3. Mechanisms of recovery of the network-driven glutamatergic activity in the presence of glutamate receptor antagonists

To understand the mechanisms of recovery of glutamatergic activity during prolonged glutamate receptor blockade, some cultures were chronically treated (on DIV4-DIV30) with various pharmacological agents (Fig. 4). The cultures then were tested in Ca^{2+} imaging, using an application of bicuculline in the presence of 1X AP5/CNQX, to see whether the bicuculline-mediated activity is expressed in these cultures, followed by a test with an acute application of the higher concentrations of AP5/CNQX, to confirm that the activity is glutamatergic. Here, 200 μ M AP5 plus 20 μ M CNQX were used (2X AP5/CNQX concentrations). The experiments were conducted in more-responsive batches (see Experimental procedures/4.4. Ca^{2+} imaging). In 1X cultures, 87% of culture fields expressed glutamatergic activity (reflected as a percentage of fields; Fig. 4). In order to determine whether inactivation of NMDA receptors or non-NMDA receptors is specifically responsible for recovery of the network-driven glutamatergic activity in the presence of receptor antagonists, we chronically treated cultures with either AP5 (100 μ M) or CNQX (10 μ M), respectively. We found that glutamatergic activity appeared in CNQX-treated cultures, although to a lesser extent than in 1X AP5/CNQX-treated cultures, but it never appeared in AP5-treated cultures. Given the role of protein kinase A (PKA), Ca^{2+} /calmodulin-dependent protein kinase (CaMK) II (CaMKII), CaMKIV, and associated down-stream signaling pathways in different forms of neuronal plasticity (Colbran and Brown, 2004; Finkbeiner and Greenberg, 1998; Greenberg and Ziff, 2001; Shaywitz and Greenberg, 1999), we tested the importance of these protein kinases in recovery of the network-driven glutamatergic activity during the receptor blockade. A chronic co-incubation of neurons in the presence of 1X AP5/CNQX and an antagonist for PKA (H89, 1 μ M) did not prevent the appearance of network-driven glutamatergic activity (Fig. 4). However, the glutamate-dependent Ca^{2+} oscillations were not detected in cultures co-incubated chronically in the presence of 1X AP5/CNQX and an antagonist for CaMKII/IV (KN-62, 2.5 μ M; Fig. 4). This suggests the role for non-NMDA receptors and CaMK-dependent signaling pathways in recovery of glutamatergic activity during chronic inactivation of ionotropic glutamate receptors.

2.4. Recovery of the network-driven glutamatergic activity at different concentrations of glutamate receptor antagonists

We tested whether the network-driven glutamatergic activity recovers in cultures at concentrations of glutamate receptor antagonists higher than 1X. Two additional groups of cultures were tested that were chronically (for 3–6 weeks, starting DIV4) treated with either 200/20 μM AP5/CNQX (2X cultures) or 500/50 μM AP5/CNQX (5X cultures). In 2X cultures, the bicuculline-mediated Ca^{2+} oscillations were observed in many neurons ($n=293$ of 482; 60.6%; tested in the continued presence of 200/20 μM AP5/CNQX) and were completely suppressed by acute administration of 300/30 μM AP5/CNQX (Fig. 5A). However, no oscillatory activity was detected during application of bicuculline in 5X cultures ($n=340$ neurons; 0%; tested in the continued presence of 500/50 μM AP5/CNQX; Fig. 5B), although the cultures looked healthy, did not demonstrate decrease in neuronal survival and were NMDA-responsive in the absence of glutamate receptor antagonists. These results suggest that the network-driven glutamatergic activity also recovers in 2X cultures, but it does not recover in the presence of 5X antagonist concentrations.

As in 1X cultures, the expression of the network-driven glutamatergic activity in 2X cultures also was field-specific (Table 1). In bicuculline-responsive fields in 2X cultures, the frequency of Ca^{2+} oscillations was 19.3 ± 3.2 oscillations/hr when measured in the presence of 200/20 μM AP5/CNQX ($n=26$; Table 1). Thus, this frequency was almost 3 times higher than that in 1X cultures when measured in the presence of 100/10 μM AP5/CNQX (i.e., 6.7 ± 0.7 oscillations/hr; $n=7$; $P=0.0007$, unpaired two-tailed Student's *t*-test; Table 1). However, in 2X cultures, decreasing the concentrations of AP5/CNQX from 200/20 μM AP5/CNQX to 100/10 μM AP5/CNQX tripled the frequency of oscillations from 7.1 ± 1.1 oscillations/hr before to 23.4 ± 4.6 oscillations/hr after the concentrations' decrease ($n=4$ fields, 103 neurons; paired Student's *t*-test; $P<0.05$; not shown). Further analysis showed that the oscillations' frequency in 2X cultures at 100/10 μM AP5/CNQX was not different from that in 1X cultures at 100/10 μM AP5/CNQX (respectively, 23.4 ± 4.6 oscillations/hr, $n=4$ fields, and 19.3 ± 3.2 oscillations/hr, $n=26$ fields; unpaired two-tailed Student's *t*-test; $P=0.631$). In bicuculline-non-responsive fields in 2X cultures, the bicuculline-mediated Ca^{2+} oscillations appeared after decreasing the concentrations of AP5/CNQX from 2X (i.e., from 200/20 μM AP5/CNQX) to 1.02 ± 0.19 X ($n=5$ fields, 115 neurons; not shown). In 5X cultures, the bicuculline-mediated Ca^{2+} oscillations appeared only after decreasing the AP5/CNQX concentrations to 2X ($n=3$ of 3 fields, 68 neurons; not shown).

2.5. Neuronal characteristics at different culture treatment conditions

To understand why the network-driven activity recovers in 1X and 2X, but not in 5X cultures, we used DIV30 control (non-treated) cultures and tested levels of the activity of ionotropic glutamate receptors in the absence and then in the presence of these three sets of concentrations of AP5/CNQX. Tests were done using the analysis of neuronal responses to glutamate (5 μM) in Ca^{2+} imaging experiments. To isolate ionotropic glutamate-mediated responses and to prevent the secondary neuronal activation, TTX and metabotropic glutamate receptor antagonists were present in all media during these tests (see Experimental procedures). The amplitude of glutamate-mediated Ca^{2+} responses was the highest in the absence of AP5/CNQX; it was reduced in the presence of 1X AP5/CNQX and was even lower in the presence of 2X AP5/CNQX; and the responses were not detectable in the presence of 5X AP5/CNQX (Fig. 6A, B). Thus, the data indicate that, in non-treated neurons, glutamate receptors can be activated by externally applied glutamate in the presence of competitive antagonists, AP5/CNQX, at 1X and 2X, but not at 5X antagonist concentrations. The data also suggest that the levels of receptor activity decrease in the following direction: no AP5/CNQX $\rightarrow$ 1X $\rightarrow$ 2X $\rightarrow$ 5X (the receptors are inactive at 5X concentrations).

We further compared a number of neuronal characteristics in cultures grown and/or incubated at different levels of glutamate receptor activity to determine which of these characteristics may be involved in recovery of the network-driven glutamatergic activity in the presence of receptor antagonists. The following four groups of hippocampal cultures were tested: (1) non-treated (control) cultures, (2) non-treated cultures in which 100/10 μ M AP5/CNQX were added for 30 min (acute AP5/CNQX), (3) 1X cultures and (4) 2X cultures (i.e., the cultures chronically treated for 3–6 weeks starting DIV4 with, respectively, 1X and 2X concentrations of AP5/CNQX); the cultures of all four groups were tested 3.5–6.5 weeks after the culture preparation.

Acute application of AP5/CNQX to non-treated neuronal cultures completely eliminated low-frequency spontaneous intracellular Ca^{2+} oscillations and spontaneous excitatory postsynaptic currents (EPSC) and/or excitatory postsynaptic potentials (EPSP) and they never were detected in 1X and 2X cultures (Fig. 1A, 7A–D, 7I–L and Table 1). Further, the average level of intracellular Ca^{2+} in neurons, the number of neurons exhibiting spontaneous Ca^{2+} transients, the frequency of spontaneous Ca^{2+} transients and the amplitude of glutamate-mediated Ca^{2+} responses were dramatically reduced by acute AP5/CNQX administration; these neuronal parameters also were significantly lower in 1X and 2X cultures than in control (with exception of frequency of spontaneous Ca^{2+} transients in 1X cultures), although they were significantly higher as compared to acute AP5/CNQX conditions (Fig. 7A–H and Table 1). In addition, some neuronal characteristics were not significantly different between the control, acute AP5/CNQX, 1X and 2X cultures, including the percentage of neurons exhibiting spontaneous action potentials, frequency of spontaneous action potentials, level of the membrane potential, input resistance, amplitude of miniature EPSC (mEPSC) and the expression of α CaMKII subunit (although the percentage of neurons firing spontaneous action potentials decreased two fold after acute AP5/CNQX administration and both 1X and 2X cultures showed slightly higher input resistance as compared to the control and acute AP5/CNQX cultures) (Fig. 7M–P, 8D, F, J, K and Table 1).

In contrast, a number of neuronal characteristics, including the average number of action potentials per step in a 20-step stimulation test (that represents an intrinsic excitability), the frequency of mEPSCs, the expression GluR1 AMPA receptor subunit, NR1 NMDA receptor subunit and β CaMKII subunit were significantly higher in 1X and/or 2X cultures as compared to the control and, in some instances (i.e., for the last three parameters), there was proportional increase between the control, 1X and 2X cultures (Fig. 8A–C, D, E, G–I, K).

3. Discussion

To our knowledge this is the first study to characterize the dynamics and mechanisms of reappearance of glutamate-dependent activity in the presence of ionotropic glutamate receptor antagonists. We demonstrate that, in mature hippocampal neurons spontaneous glutamate-dependent synaptic activity (i.e., spontaneous Ca^{2+} oscillations, EPSPs and EPSCs) is suppressed by acute blockade of glutamate receptors and remains suppressed after the chronic blockade. However, the network-driven glutamate-dependent activity, which is revealed during the hyperexcitable states and also is suppressed by acute blockade of glutamate receptors, does appear in neurons after the chronic glutamate receptor blockade. In neurons subjected to the prolonged inactivation of glutamate receptors, this network-driven activity is synaptically-mediated (as suppressed by the blockade of action potentials with TTX) and is represented in recordings by synchronized giant EPSPs and large intracellular Ca^{2+} oscillations. Given that the network-driven glutamatergic activity also is expressed in developing hippocampal neurons (Arnold et al., 2005), we consider the observed phenomenon as recovery of the network-driven glutamatergic activity in conditions of the prolonged inactivation of glutamate receptors.

We believe there are two possible explanations for recovery of the network-driven glutamatergic activity in the presence of glutamate receptor antagonists. Our data demonstrate that relative to control or acute AP5/CNQX, some neuronal parameters are increased in cultures after the chronic inactivation of glutamate receptors. These include increases in the expression of GluR1, NR1 and β CaMKII subunits, mEPSC frequency, and neuronal excitability (evident from a 20-step stimulation test). Some of these parameters likely reflect changes on the presynaptic site (e.g. mEPSC frequency), others may represent the postsynaptic changes (e.g. receptor expression, but also mEPSC frequency, which may happen if the number of synaptic connections increases after the chronic glutamate receptor blockade). Previous studies from other laboratories in neuronal cultures, also revealed an increase in both presynaptic and postsynaptic components during the prolonged (hours to days) blockade of glutamate receptors or action potentials, including an increase in the bouton size (Murthy et al., 2001), readily releasable pool of vesicles (Murthy et al., 2001), presynaptic release rate (Bacci et al., 2001), amplitude of mEPSCs (Turrigiano et al., 1998), and expression of vesicular glutamate transporters (De Gois et al., 2005) and glutamate receptor subunits (Thiagarajan et al., 2005). Therefore, the first possibility is that the up-regulation of a number of presynaptic and postsynaptic parameters during chronic inactivation of glutamate receptors, becomes sufficient to overcome the effects of the receptor blockade, so that the network-driven glutamatergic activity recovers in the presence of receptor antagonists.

Alternatively, expression of a novel, AP5/CNQX-resistant glutamate receptor subunit, may also be possible. In our experiments, the network-driven glutamatergic activity was induced and mediated by non-NMDA receptors, suggesting possible alternate regulation of a novel AMPA or kainate receptor subunit. A previous study has shown that an alternate splice variant of the NMDA receptor is trafficked to the synaptic membrane after 1 hr of NMDA receptor blockade, resulting in a modest recovery of the NMDA receptor-dependent current in the presence of the NMDA receptor antagonist (Mu et al., 2003). However, since in 1X and 2X cultures the network-driven activity is suppressed by the higher AP5/CNQX concentrations and since 5X cultures do not exhibit the network-driven glutamatergic activity in the presence of 500/50 μ M AP5/CNQX, the former possibility seems more likely. Change in the number of synaptic terminals also is unlikely responsible for recovery of the activity as in the treatment conditions that are nearly identical to ours (3 weeks; 100 μ M/20 μ M AP5/CNQX) the number of synapses in hippocampal cultures remains unchanged (Bacci et al., 2001).

The chronic inactivation of non-NMDA receptors is sufficient to induce network-driven glutamatergic activity and this activity is suppressed by higher concentrations of non-NMDA receptor antagonists. Further, the expression of GluR1 receptor subunit is up-regulated in both 1X and 2X cultures. Additionally, β CaMKII (but not α CaMKII) subunit is upregulated in both groups of chronically treated cultures and recovery of the network-driven glutamatergic activity is prevented by CaMKII/IV inactivation. This suggests that non-NMDA receptors and CaMK-dependent signaling pathways (presumably including Ca^{2+} -permeable GluR1-containing AMPA receptors and β CaMKII subunit) play an important role in the recovery. Because in hippocampal neurons, CaMKII and non-NMDA receptors also have been implied in the homeostatic regulation of glutamatergic mEPSCs (Thiagarajan et al., 2005; Thiagarajan et al., 2002), recovery of the network-driven glutamatergic activity in 1X and 2X chronically treated cultures perhaps may represent a form of such homeostatic processes. Compared to those previous studies, however, we did not find a significant increase in the amplitude of mEPSCs in hippocampal neurons during glutamate receptor blockade. One possibility for this difference is that we used the long-term inactivation of both NMDA and non-NMDA receptors, while only non-NMDA receptor blockade for the relatively short-term period (24–48 hrs) was used in the studies of homeostatic synaptic plasticity (Thiagarajan et al., 2005; Thiagarajan et al., 2002). Another study, which used experimental conditions similar to ours (Bacci et al., 2001), i.e., the prolonged treatment (for 3 weeks, starting DIV4) of cultured hippocampal

neurons with 100 μ M AP5 and 20 μ M CNQX, also did not reveal increases in the amplitude of mEPSCs, but demonstrated increases in the mEPSCs' frequency.

In our study, the network-driven glutamatergic activity does not recover in cultures subjected to selective NMDA receptor blockade and is not suppressed by the higher concentration of the NMDA receptor antagonist. This suggests that NMDA receptors do not contribute directly to the recovery and generation of this activity. Some contribution of NMDA receptors, however, is evident from the more efficient recovery of the network-driven glutamatergic activity in AP5/CNQX-treated than in CNQX only-treated cultures. Additionally, the frequency of network-driven glutamatergic activity increases after removal of the NMDA receptor antagonist from neurons and the expression of NR1 NMDA receptor subunit increases in both 1X and 2X cultures, presumably reflecting some role of NMDA receptors in modulation of the network-driven (largely non-NMDA receptor-dependent) activity.

Recovery of the network-driven glutamatergic activity is faster in mature than in immature cultures. This could be due to the fact that the synaptic contacts are already established in mature cultures and are still developing in the immature cultures (van den Pol et al., 1998). Moreover, because the GABA_AR-mediated activity is largely excitatory during early cell culture development (Ganguly et al., 2001; Obrietan and van den Pol, 1995), the network-driven glutamatergic activity may not be revealed: the application of bicuculline will result in neuronal inhibition, not disinhibition until about DIV10. Additionally, the network-driven glutamatergic activity is detected in cultures after 2 months of the continued glutamate receptor blockade (Fig. 2C), suggesting that the glutamatergic up-regulation sustains during the prolonged time periods.

It is interesting to note that the network-driven glutamatergic activity recovers at two increasing sets of concentrations of AP5/CNQX, 1X and 2X, and it does not recover in 5X-treated cultures at 5X. Further, the results indicate that the level of glutamate receptor activity decreases in the following direction: no AP5/CNQX $\rightarrow$ 1X $\rightarrow$ 2X, and the receptors are inactive at 5X concentrations. Altogether the data suggest that some level of the receptor activity is necessary for the network-driven activity recovery to occur. In this respect, it may not be surprising that the number of neuronal parameters (e.g. the expression of GluR1, NR1 and β CaMKII) are up-regulated proportionally to the level of glutamate receptor inactivity (Fig. 8). This proportional increase presumably is necessary for recovery of the elements of glutamatergic transmission at the increasing levels of glutamate receptor inactivity. However, even if the network attempts to compensate for decrease in glutamate-dependent transmission, there is an effect of saturation. When the receptors are completely inactive, the compensatory changes (even if occur in 5X cultures) presumably are insufficient for recovery of the network-driven activity.

In addition, the level of inactivity of glutamate receptors also is the factor which determines the frequency of the recovered network-driven glutamatergic activity. For example, the frequency of bicuculline-mediated Ca²⁺ oscillations is 118.3 $\pm$ 32.7 oscillations/hr in the control, 19.3 $\pm$ 3.2 oscillations/hr in 1X cultures and 6.7 $\pm$ 0.7 oscillations/hr in 2X cultures. Moreover, when in 2X cultures the concentration of the antagonists is reduced to 1X, the frequency of Ca²⁺ oscillations increases (23.4 $\pm$ 4.6 oscillations/hr) and becomes similar to that in 1X cultures at 1X. The activity is absent (i.e., frequency is zero) in 5X cultures at 5X that also correlates with the idea that the level of the inactivity of glutamate receptors determines the frequency of the network-driven activity.

Although, when tested in the presence of the corresponding antagonist concentrations, the network-driven activity does not appear in bicuculline-non-responsive fields in 1X and 2X cultures and also in 5X cultures, there is some degree of compensation for the glutamate receptor inactivity in these fields/cultures too. This compensation is manifested in appearance

of the network-driven glutamatergic activity at about 0.5X in 1X cultures, 1X in 2X cultures and 2X in 5X cultures, while only 0.3X is sufficient to completely suppress the bicuculline-mediated glutamatergic activity in the control cultures. In addition, the fact that network-driven glutamatergic activity reappears in 5X cultures at 2X suggests that saturation for compensation, which is discussed above, presumably occurs at the antagonist concentrations just above 2X (i.e., between 2X and 3X; see Experimental procedures).

The results indicating that three neuronal parameters increase proportionally to the level of glutamate receptor inactivity have an additional meaning. They suggest that not only glutamate receptor inactivity induces increases in these parameters, but when the increases are sufficient to compensate for the receptor inactivity (so that some elements of glutamatergic activity may reappear), there is a subsequent cessation of increases. As a result, the new levels of these neuronal parameters are formed that correspond to the levels of inactivity of glutamate receptors.

What are the signals/mechanisms that cause the cessation of increases in these parameters? One possibility is extant glutamatergic synaptic activity. In our experiments, however, we never saw any evidence of spontaneous glutamate-dependent Ca^{2+} oscillations, EPSCs or EPSPs in 1X and 2X cultures in the presence of glutamate receptor antagonists. This suggests that the putative recovery of glutamate-dependent synaptic activity, per se, is not the signal for a neuron to cease further increases in these parameters. Additionally, no statistical difference in some neuronal parameters (including the resting membrane potential, input resistance, number of neurons with spontaneous action potentials and frequency of spontaneous action potentials) between the control, acute AP5/CNQX, 1X and 2X cultures was detected, suggesting that the changes in these parameters may be not critical for the cessation (and, perhaps, initiation too) of increases. Some parameters, however, which decreased after acute AP5/CNQX administration, were significantly higher in both 1X and 2X cultures as compared to the acute AP5/CNQX conditions. This includes average level of intracellular Ca^{2+} , the number of neurons with spontaneous Ca^{2+} transients, frequency of spontaneous Ca^{2+} transients and the amplitude of glutamate-mediated Ca^{2+} responses. We hypothesize that some of these parameters (e.g. the average level of intracellular Ca^{2+} and/or the pattern of intrinsic Ca^{2+} activity) are responsible for the cessation of changes. Future studies in hippocampal neurons *in vitro* and *in vivo* will confirm or refute this hypothesis.

Our previous work demonstrated that the chronic blockade of ionotropic glutamate receptors induces the expression of spontaneous cholinergic EPSCs and EPSPs and ACh-dependent intracellular Ca^{2+} activity in hypothalamic and cerebellar neuronal cultures, but not in cortical cultures (Belousov et al., 2001; Liu et al., 2008). The data also suggested an important role for NMDA receptors in the development of ACh-dependent activity during glutamate receptor blockade. We postulated that glutamate-dependent cholinergic regulation contributes to the neurotransmitter phenotype specification in developing neurons, represents the form of compensation for the absence of glutamatergic excitatory activity in both developing and mature neurons, and is region-specific.

As in the hypothalamus (Belousov et al., 2002; Belousov et al., 2001; Liu et al., 2008), a small number of cholinergic neurons (i.e., immunopositive for choline acetyltransferase) is expressed in the rat hippocampus and cultured hippocampal neurons express cholinergic receptors (Frotscher et al., 2000; Kawai et al., 2002; Volpicelli and Levey, 2004). However, in the present study, neither spontaneous cholinergic EPSCs/EPSPs nor ACh-dependent Ca^{2+} activity appear in hippocampal neurons after prolonged glutamate-receptor blockade, supporting the region-specific character for glutamate-dependent cholinergic regulation. Instead, the network-driven glutamatergic activity recovers and non-NMDA receptors play an important role in such a recovery. Altogether, the data suggest that in different regions of the nervous system

compensation for the decreased glutamatergic transmission may exist in different forms and involve different mechanisms, i.e., NMDA receptor-dependent mechanisms for cholinergic up-regulation in the hypothalamus and non-NMDA receptor-dependent mechanisms for glutamatergic up-regulation in the hippocampus. However, the resulting effect of these mechanisms is the recovery of excitatory network activity.

Previous studies showed that while CNQX, a competitive AMPA/kainate receptor antagonist, transiently prevents epileptiform discharges in rats and in brain slices, it fails to prevent the epileptiform activity over time (Mazarati and Wasterlain, 1999; Zhang et al., 1994). Our data demonstrate recovery of glutamate-dependent network activity in the presence of glutamate receptor antagonists, and CNQX particularly. Agents that reduce the activity of AMPA/kainate receptors are used or considered for the treatment of a number of neurological disorders such as epilepsy, stroke and Parkinson's disease. Some of such agents are talampanel, topiramate and zonampanel (Belayev et al., 2001; Ferro and Davalos, 2006; Latini et al., 2008; Minematsu et al., 2005; also see <http://clinicaltrials.gov/>). Recovery of glutamatergic activity (or its elements) may be another factor for consideration in use of glutamate receptor antagonists in clinical conditions.

4. Experimental procedures

4.1. Animals

The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23; revised 1996). The formal approval of the described experiments has been obtained from the animal review board of Tulane University (New Orleans, LA). All efforts were made to minimize the number of animals used and their suffering.

4.2. Culture preparation and treatment

Pregnant Sprague-Dawley rats were anesthetized with nembutal and neuronal cultures were prepared as described (Belousov et al., 2001) from embryonic day 18–19 hippocampus. Neurons were plated on glass coverslips and raised in glutamate- and glutamine-free minimal essential medium (Invitrogen, Carlsbad, CA, USA) with supplements and cytosine β -D-arabinofuranoside (1 μ M) (Belousov et al., 2001). The following concentrations of AP5 (an NMDA receptor antagonist) and CNQX (a non-NMDA receptor antagonist) were used in most of the tests: 100 μ M AP5 plus 10 μ M CNQX (100/10 μ M; *1X AP5/CNQX concentrations*); 200 μ M AP5 plus 20 μ M CNQX (200/20 μ M; *2X AP5/CNQX concentrations*); 500 μ M AP5 plus 50 μ M CNQX (500/50 μ M; *5X AP5/CNQX concentrations*). Most of experiments were conducted in the following four groups of neuronal cultures: (1) non-treated (*control*) cultures, (2) non-treated cultures in which 100/10 μ M AP5/CNQX were added acutely for 30 min (*acute AP5/CNQX cultures*), (3) cultures chronically treated with 100/10 μ M AP5/CNQX (*1X cultures*), and (4) cultures chronically treated with 200/20 μ M AP5/CNQX (*2X cultures*). Additionally, a number of experiments was done in cultures that were chronically treated with 500/50 μ M AP5/CNQX (*5X cultures*) or in 1X cultures that were co-treated with various pharmacological agents, as described in text of the paper. The treatments were done starting DIV4 and in most of the experiments lasted for 3–6 weeks. Therefore, most of the cultures that were tested in this study can be considered as mature cultures (Cline, 2001; Dotti et al., 1988; van den Pol et al., 1998). A combined blockade of both NMDA and non-NMDA receptors was used in many experiments in this study because it is most efficient in the induction of cholinergic properties in hypothalamic neurons (Belousov et al., 2002; Belousov et al., 2001) and in recovery of network-driven glutamatergic activity in hippocampal neurons (present work). Cell survival was estimated by counting the number of neurons per microscope field (20X magnification) as described previously (Belousov et al., 2001) and none of the

chronic treatments reduced neuronal survival as compared to the control. We do not exclude that high concentrations of AP5/CNQX (e.g., 5X) may have some non-specific effects on cultured neurons. However, the facts that 5X cultures do not demonstrate neuronal cell death, do not have visual morphological differences from other culture groups, and are responsive (e.g., to NMDA) suggest that the chronic treatment with 5X antagonist concentrations have rather specific effects. The culture medium was changed twice a week.

4.3. Electrophysiology

Standard bathing solution contained: 135.5 mM NaCl, 25 mM NaHCO₃, 5 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 14 mM glucose and 1 μM glycine (pH 7.3, 320 mOsm, 22°C). Pipette solution contained: 145 mM KMeSO₄, 10 mM HEPES, 2 mM MgCl₂, 0.1 mM CaCl₂, 1.1 mM EGTA, 2 mM Na-ATP and 0.3 mM Na-GTP (pH 7.2, 310 mOsm, 3–6 MΩ electrode resistance). In both electrophysiology and Ca²⁺ imaging experiments, a slightly elevated KCl (5 mM) was used in all testing solutions to increase the probability of detection of low-amplitude electrophysiological and calcium events. The whole-cell current-clamp or discontinuous voltage-clamp recordings were made with an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, USA) as described (Belousov et al., 2001). Data were monitored using a PC computer and pCLAMP7 software (Axon Instruments) and analyzed off-line with Igor Pro (WaveMetrics, Lake Oswego, OR, USA) and InStat (GraphPad Software, San Diego, CA, USA) software. A 20-step stimulation test (square-wave 5 pA steps of current, 0–95 pA, 100 ms) was conducted from the resting membrane potential or, if neurons were depolarized and spontaneously firing, from the average equivalent of the resting membrane potential obtained by the negative current injection. This test represents an increased intrinsic excitability (Brager and Johnston, 2007) and was used in the present study to determine whether the neurons after chronic glutamate receptor blockade are more excitable (i.e., fire more action potentials during cell depolarization) than control neurons. A majority (>90%) of cultured embryonic hippocampal neurons were presumed to be excitatory based on previous studies (Benson et al., 1994). Spontaneous EPSCs and EPSPs were recorded in the absence of AP5/CNQX at, respectively, the holding potential of –60 mV and the resting membrane potential. mEPSCs were recorded in the absence of AP5/CNQX, but in the presence of TTX (2 μM; a voltage-gated sodium channel blocker) and bicuculline (50 μM; GABA_AR antagonist) at the holding potential of –60 mV. For cell perfusion and to change from one solution to another, a flow pipe perfusion system was used (Belousov et al., 2001) that allows delivery of the newly applied solution to the tested cell in less than a half of second. For each cell, the following sequence of procedures was used: 1) obtaining the stable whole-cell contact in voltage-clamp mode; 2) conducting the background recording to determine the expression of spontaneous EPSCs (1–2 min); 3) running pre-programmed voltage-step protocol to determine input resistance; 4) switching to current-clamp mode to determine characteristics of the resting membrane potential, action potentials, and spontaneous EPSPs (1–2 min recording); and 5) conducting a pre-programmed 20-step stimulation test protocol.

4.4. Ca²⁺ imaging

Fura-2 calcium imaging was performed in cultures as described (Belousov et al., 2001). Cells were tested using a laminar style 180-μl perfusion chamber (Warner Instruments; Hamden, CT) that allows for rapid (5–10 sec) and complete change in the medium. Neurons were perfused at the constant flow rate of 2 ml/min, but at the higher rate during drug washouts (e.g. to washout glutamate, NMDA, etc.). Perfusion solution contained: 137 mM NaCl, 25 mM glucose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl₂, 3 mM CaCl₂ and 1 μM glycine (pH 7.4, 22°C). Calcium standards from Invitrogen were used to calibrate the imaging system (Belousov et al., 2001). Calibrated Ca²⁺ data were analyzed with Igor Pro and InStat. Ca²⁺ levels varied from 30 nM to 120 nM, therefore experiments examining baseline Ca²⁺ levels, frequency of Ca²⁺ transients and glutamate sensitivity in four main groups of cultures (see above) were

paired to specific calibrations. Recordings were done from cell bodies of neurons, which usually were rounded, had small size (~20 μm) and were easily distinguished from astrocytes which did not have a clear-cut cell body, were spread, and had a bigger size (40–100 μm).

4.4.1. Types of calcium activity—We observed several different types of Ca^{2+} activity in our experiments and defined them as follows: (1) “Spontaneous Ca^{2+} transients” were not synchronized between different neurons in the same microscope field, were expressed in all four main groups of cultures, had an amplitude in the range of 10–50 nM Ca^{2+} and were not completely suppressed by AP5/CNQX (suggesting intrinsic mechanisms for their generation); (2) “Spontaneous Ca^{2+} oscillations” were distinguished from spontaneous Ca^{2+} transients by amplitude (75–150 nM Ca^{2+}), synchronization (all neurons in the field), frequency (comparatively low), expression (found only in the control group of cultures), and presumptive glutamatergic origin (as all were completely suppressed by AP5/CNQX); (3) “Bicuculline-mediated Ca^{2+} oscillations” (or also “network-driven glutamatergic activity”) were distinguished by their appearance (detected only during bicuculline application), amplitude (large; 100–1000 nM Ca^{2+}), synchronization (all neurons in the field), expression (found in the control, 1X and 2X, but not in acute AP5/CNQX or 5X cultures), and sensitivity to AP5/CNQX or to the higher concentrations of AP5/CNQX; and (4) “Glutamate-mediated Ca^{2+} increases” were induced in neurons by application of glutamate.

4.4.2. Detection of calcium events—Spontaneous Ca^{2+} transients were detected using Igor Pro by finding the interpolated peak and time where the wave derivative crossed zero concomitant with a minimum height of >99% of the confidence interval from the average Ca^{2+} level in a control baseline recording. The frequency and amplitude of spontaneous Ca^{2+} oscillations and transients and the level of intracellular Ca^{2+} were measured during 500 s recordings. The occurrence of bicuculline-mediated Ca^{2+} oscillations was recognized if at least one oscillation appeared within 1000 s after the beginning of bicuculline administration and two or more other oscillations appeared after that (in some instances, the waiting period was up to 90 min, i.e., in 2X cultures). A neuron was considered as responding to a pharmacological agent (e.g. glutamate, bicuculline, etc.) if during the agent application either Ca^{2+} increased by >10 nM from the initial background level (and then decreased after washout) or large intracellular Ca^{2+} oscillations appeared (usually > 100 nM in amplitude). Responses of neurons to glutamate were tested in the presence of TTX (2 μM) alone or TTX plus three metabotropic glutamate receptor antagonists (AIDA, 100 μM ; EGLU, 100 μM ; and MSOP, 100 μM) as indicated in the text and/or figure legends. In some experiments, NMDA-containing, but Mg^{2+} -free solution was used to confirm that cells were healthy and responsive.

4.4.3. Responsiveness of cultures to bicuculline—The incidence of responsiveness of hippocampal cultures to bicuculline was generally dependent on individual culturing events (e.g. a single collection and plating of embryonic tissue from a pregnant rat; usually plated on 24–30 coverslips). Therefore, in 1X, 2X and 5X cultures, an initial test was done to determine the responsiveness of culture batches. The test was conducted in 5–8 randomly chosen microscope fields from 1–2 coverslips and included the analysis of bicuculline-mediated Ca^{2+} oscillations induced in the presence of the corresponding concentrations of AP5/CNQX (e.g. 1X AP5/CNQX concentrations in 1X cultures, etc.). If, during this test, about 80% of fields were bicuculline-responsive, most of the fields in other coverslips from this batch also were responsive. Such batches were considered as “more-responsive” and contained (on average) ~80% of bicuculline-responsive and ~20% of bicuculline-non-responsive fields. Batches in which only 10–30% of fields were bicuculline-responsive were considered as “less-responsive”. The more-responsive and less-responsive batches did not appear different from each other in a light microscope. The bicuculline-responsive and non-responsive fields (which could be found in the same coverslip) also were not visually different. In this study, the more-

responsive batches were used to determine timing of development of the network-driven glutamatergic activity in the presence of glutamate receptor antagonists and effects of inhibitors for CaMKII/IV and PKA on development of this activity. However, the results from both more-responsive and less-responsive batches are summarized in the table representing the average number of the bicuculline-responsive and non-responsive fields in 1X and 2X cultures. To determine whether the bicuculline-mediated activity appears at reduced concentration of AP5/CNQX in bicuculline-non-responsive fields/batches, the concentrations were reduced by 100 s intervals in the following sequences: 1X → 0.7X → 0.3X → 0X in 1X and acute AP5/CNQX cultures; 2X → 1.7X → 1.3X → 1X → 0.7X → 0.3X → 0X in 2X cultures; and 5X → 4X → 3X → 2X → 1.7X in 5X cultures (all numbers are coefficients of 1X AP5/CNQX). The concentrations were reduced until appearance of the first large Ca^{2+} spike and the average concentration at which the spike appeared was calculated for each group.

4.5. Western blots

Protein was obtained by homogenizing cells in a lysis buffer as described (Arumugam et al., 2005). Total protein was determined using the Bio-Rad DC protein assay method. Thirty μg of protein were loaded in each lane, resolved by 10% SDS-PAGE and transferred to 0.2 mm Polyvinylidene difluoride membrane. The membrane was blocked in TBS and then probed with a primary antibody. The following primary antibodies were used: rabbit anti-GluR1 (1.75 $\mu\text{g}/\text{ml}$, Chemicon, Temecula, CA, USA, cat.# AB1504), rabbit anti-NR1 (1 $\mu\text{g}/\text{ml}$, Chemicon, cat.# AB1516), mouse anti- β -actin (0.2 $\mu\text{g}/\text{ml}$, Chemicon, cat.# AB8226), mouse anti- βCaMKII (2 $\mu\text{g}/\text{ml}$, Abcam, Cambridge, MA, USA, cat.# AB2725) and mouse anti- αCaMKII subunit (2 $\mu\text{g}/\text{ml}$, Abcam, cat.# AB22131). The primary antibody was visualized with goat alkaline phosphatase-conjugated anti-rabbit antibody (1:1,000–1,500, Vector Laboratories, Burlingame, CA, USA, cat.# AP-1000), goat horseradish peroxidase-conjugated anti-rabbit antibody (1:2,000–10,000, Invitrogen, cat.# 62–1820), horse alkaline phosphatase-conjugated anti-mouse antibody (1:1,000–1,500, Vector Laboratories, cat.# AP-2000) or rabbit horseradish peroxidase-conjugated anti-mouse antibody (1:2,000–10,000, Invitrogen, cat.# 61–6020). Signals were enhanced using an Immuno-Star AP chemiluminescence substrate (Bio-Rad, Hercules, CA, USA) or Lumigen PS-3 (Amersham Biosciences, Piscataway, NJ, USA). Band optical density was determined by analyzing 16 bit gray scale image mean pixel values in OpenLab Software (Improvision, Lexington, MA, USA). Optical density was calculated as $\log_{10}(p/\text{mean})$, where p was the background and mean was the signal. Actin served as the loading control and was never different between the different experimental conditions. Because some optical density values were zero in control cultures, data were normalized and compared relative to 2X cultures (set at 1.0).

4.6. Drugs and chemicals

All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Tocris (Ellisville, MO, USA) unless otherwise specified.

4.7. Statistical analysis

Some Ca^{2+} imaging, electrophysiological, and western blot tests presented in this paper include statistical data from cultures ranging from 3.5 to 6.5 week old. However, each experimental group in each test had the matching sister culture in all other experimental groups in that particular test. Data were analyzed using the two-tailed Student's t -test (paired when possible), ANOVA with post hoc Tukey, repeated measures ANOVA, Fisher's exact probability test and InStat software. Statistical data are reported as mean $\pm$ SE for the number of samples indicated.

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Abbreviations

1X	100 μ M/10 μ M AP5/CNQX concentrations or chronically treated culture condition
2X	200 μ M/20 μ M AP5/CNQX concentrations or chronically treated culture condition
5X	500 μ M/50 μ M AP5/CNQX concentrations or chronically treated culture condition
ACh	acetylcholine
AP5	D,L-2-amino-5-phosphonovalerate
CaMK	Ca ²⁺ /calmodulin-dependent protein kinases
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DIV	day <i>in vitro</i>
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA_AR	γ -aminobutyric acid A receptor
mEPSC	miniature excitatory postsynaptic current
NMDA	N-methyl-D-aspartate
PKA	cAMP-dependent protein kinase
TTX	tetrodotoxin

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Fig. 1.

ACh-dependent activity does not develop in hippocampal neuronal cultures after chronic glutamate receptor blockade. Representative Ca²⁺ imaging recordings are shown. (A) Application of bicuculline (BIC) to this non-treated neuron induces large amplitude intracellular Ca²⁺ oscillations that are glutamatergic in nature and are suppressed by AP5/CNQX (also note the presence of low-frequency spontaneous Ca²⁺ oscillations in the background recording that are AP5/CNQX-sensitive too). (B) No bicuculline-mediated Ca²⁺ oscillations are detected in another non-treated neuron shortly after AP5/CNQX administration. (C) The bicuculline-mediated oscillations are expressed in 1X cultures in the continued presence of 100/10 μ M AP5/CNQX. These oscillations are not suppressed by ACh

receptor antagonists, atropine plus mecamylamine (A+M; 100 μ M each) and, therefore, they are not cholinergic in origin. Here and in the following figures, applications of drugs are indicated by bars or arrowed bars. Concentrations: none AP5/CNQX, 0/0 μ M; 1X AP5/CNQX, 100/10 μ M; BIC, 50 μ M.

Fig. 2.

Occurrence of the bicuculline-mediated activity in hippocampal neurons after chronic glutamate receptor blockade. Data are from 1X cultures. (A) Simultaneous Ca^{2+} imaging recordings from ten neurons illustrate synchronous Ca^{2+} oscillations. (B) Dual current-clamp recordings also illustrate the synchronized giant EPSPs; boxed are the same recordings, but with a different scale. Recordings in A and B were done in the presence of 1X AP5/CNQX and bicuculline. (C–D) Expression of bicuculline-mediated Ca^{2+} activity in cultures treated with 1X AP5/CNQX starting from DIV4 (C) and DIV30 (D). Cells were tested on day(s) *in vitro* as indicated in graphs using Ca^{2+} imaging and the analysis of bicuculline-mediated Ca^{2+} oscillations induced in the presence of 1X AP5/CNQX. In both graphs, data are shown

as percentage of fields exhibiting bicuculline-mediated Ca^{2+} oscillations; n=4–8 fields (>130 neurons) in each test.

Fig. 3.

Non-NMDA receptors are responsible for generation of the network-driven activity in hippocampal cultures after chronic glutamate receptor blockade. Representative Ca^{2+} imaging recordings (A–E) and statistical analysis (F) in 1X cultures are shown. (A–E) The bicuculline-mediated Ca^{2+} activity is not affected by the doubled concentration of AP5 (A), is increased after AP5 removal (B), is suppressed by the doubled concentration of AP5/CNQX (C) or CNQX only (D), and is increased after CNQX removal (E). (F) Graph represents the frequency of bicuculline-mediated Ca^{2+} oscillations prior to (open columns) and after (filled columns) the agent application; (+) indicates the agents added to the solution; pluses or minuses in the box indicate, respectively, the presence or absence of 1X AP5/CNQX in the agent-containing solution. Data are shown as mean $\pm$ SE; $n=4-6$ bicuculline-responsive fields ($n>150$ neurons for each test). Statistical analysis was done for each group between the two conditions (before and after the agent application): * $P<0.05$, ** $P<0.01$ (paired Student's *t*-test). Concentrations (also see figure 1): none AP5 or none CNQX, 0 μM ; 1X AP5, 100 μM ; 2X AP5, 200 μM ; 1X

CNQX, 10 μ M; 2X CNQX, 20 μ M; 2X AP5/CNQX, 200/20 μ M; carbenoxolone (Carb), 20 μ M; DNQX, 10 μ M; TTX, 2 μ M. A+M, atropine plus mecamylamine.

Fig. 4.

Expression of the network-driven glutamatergic activity in cultures under different treatment conditions. Graph represents the results from Ca^{2+} imaging experiments. The network-driven glutamatergic activity was detected in cultures treated with 1X AP5/CNQX or 1X CNQX, but not in cultures treated with 1X AP5. In 1X cultures, chronic inactivation of PKA (with H89, 1 μM) did not affect expression of the network-driven glutamatergic activity, however, chronic inactivation of CaMKII/IV (with KN-62, 2.5 μM) prevented the activity expression. Data are shown as percentage of fields exhibiting glutamate-dependent Ca^{2+} oscillations; $n=4-8$ fields (>130 neurons) in each test. Concentrations: see figures 1 and 3.

Fig. 5.

Recovery of the network-driven glutamatergic activity in cultures grown at different concentrations of glutamate receptor antagonists. Representative Ca^{2+} imaging recordings are shown. (A–B) The network-driven glutamatergic activity recovers in 2X cultures (A), but not in 5X cultures (B). Concentrations (also see figures 1 and 3): 3X AP5/CNQX, 300/30 μM ; 5X AP5/CNQX, 500/50 μM . NMDA-containing (10 μM), but Mg^{2+} -free solution in B was used to confirm that the cells were healthy and responsive.

Fig. 6. Glutamate-mediated responses in non-treated hippocampal neurons at different levels of glutamate-receptor blockade. (A) Simultaneous Ca^{2+} imaging recordings from three neurons in the same microscope field demonstrate responses to glutamate (GLU; 5 μM) at different concentrations of competitive ionotropic glutamate receptor antagonists, AP5/CNQX. The amplitude of responses decreases with increase in concentration of the antagonists. (B) Graph represents statistical analysis of amplitude of responses. Data are shown as mean $\pm$ SE; n=87 neurons (in 3 microscope fields). Statistical significance (*** $P < 0.001$) is shown relative to responses in the absence of the antagonists ("none AP5/CNQX") and is estimated using one-way ANOVA with post-hoc Tukey. In parentheses, percentage of neurons responding to glutamate. All media contained TTX and metabotropic glutamate receptor antagonists (see Experimental procedures).

Fig. 7.

Characteristics of neuronal activity in hippocampal cultures grown and/or incubated at different levels of glutamate receptor activity. Columns represent the data from (left to right) control, acute AP5/CNQX, 1X and 2X cultures. The traces illustrate representative recordings from Ca^{2+} imaging (A–H), voltage-clamp (I–L) and current-clamp (M–P) experiments. (A–D) Low-frequency spontaneous Ca^{2+} oscillations were detected in non-treated neurons (indicated by vertical arrows in A), were suppressed by acute AP5/CNQX (B) and never appeared in 1X and 2X cultures (C, D). Spontaneous Ca^{2+} transients were expressed in all culture groups (indicated by arrowheads in A–D), but their frequency was the lowest after acute AP5/CNQX administration (B). (E–H) The neurons were glutamate-responsive (GLU, 5 μM) even in the presence of competitive antagonists, AP5/CNQX (F–H), however, the amplitude of responses was the lowest in acute AP5/CNQX conditions (F). (I–L) EPSCs were detected in non-treated cultures (I), were suppressed by acute AP5/CNQX (J) and never appeared in 1X and 2X cultures (K, L). (M–P) No significant difference between the culture groups in the frequency of action potentials was established. Recordings were made in the absence of AP5/CNQX in the control cultures and in the presence of 100/10 μM AP5/CNQX in acute AP5/CNQX and 1X cultures

and 200/20 μM AP5/CNQX in 2X cultures. In E–H, all media contained 2 μM TTX. In I–L, holding potential was -60 mV.

Fig. 8.

The expression of a number of neuronal parameters increases in hippocampal cultures after chronic glutamate receptor blockade. (A–C) Illustrated are recordings (A), data from 4 representative neurons with fitting curves (B) and data analysis (C) from 20-step (5 pA, 0–95 pA, 100 ms) stimulation tests. Statistical significance in C was calculated relative to the control group (repeated measures ANOVA; * $P < 0.05$; ** $P < 0.01$); $n = 13$ –14 neurons in each group. The recordings were made in the absence or presence of the corresponding concentrations of AP5/CNQX. (D–F) Representative electrophysiological recordings (D) and statistical analysis of the frequency (E) and amplitude (F) of glutamatergic mEPSCs are shown. Significance of differences was calculated relative to the control group (ANOVA with post hoc Tukey; * $P < 0.05$); $n = 10$ –13 neurons in each group. None media contained AP5/CNQX, but all included TTX (2 μ M) and bicuculline (50 μ M). (G–K) Expression of GluR1 (G), NR1 (H), β CaMKII (I) and α CaMKII (J) subunits and statistical data (K) are presented. In western blots, actin was used as loading control. Because some optical density values were zero in the control cultures, data were normalized relative to 2X cultures (set at 1.0). Significance of difference is calculated using repeated measures ANOVA and is shown relative to 2X (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and 1X (# $P < 0.05$, ### $P < 0.001$); $n = 12$ for actin and $n = 4$ for each of other tests. In all graphs (C, E, F, K), data are presented as mean $\pm$ SE.

Characteristics of neuronal activity in hippocampal cultures grown and/or incubated at different levels of glutamate receptor activity

Table 1

Culture condition	Control	Acute AP5/CNQX	1X	2X
Neuronal characteristics				
Neurons with bicuculline-mediated Ca^{2+} oscillations in the number of fields ^{a,e,f}	62 of 62 (100%) in 3 of 3 fields	0 of 477 (0%) in 0 of 16 fields $P<0.001^c$	724 of 1201 (60.3%) in 26 of 43 fields NS^c , $P<0.001^d$	293 of 482 (60.6%) in 7 of 12 fields NS^c , $P<0.001^d$
Frequency of bicuculline-mediated Ca^{2+} oscillations, oscillations/hr ^{b,f}	118.3 ± 32.7 (n=3 fields)	0 $P<0.001^c$ (n=16 fields)	19.3±3.2 $P<0.001^c$, $P<0.0001^d$ (n=26 fields)	6.7±0.7 $P<0.001^c$, $P<0.0001^d$ (n=7 fields)
Neurons with spontaneous Ca^{2+} oscillations ^a	243 of 243 (100%)	0 of 181 (0%) $P<0.0001^c$	0 of 160 (0%) $P<0.0001^c$, NS^d	0 of 132 (0%) $P<0.0001^c$, NS^d
Frequency of spontaneous Ca^{2+} oscillations, oscillations/hr ^b	18.0±1.6 (n=243 neurons)	0 $P<0.001^c$ (n=181 neurons)	0 $P<0.001^c$, NS^d (n=160 neurons)	0 $P<0.001^c$, NS^d (n=132 neurons)
Neurons with spontaneous EPSCs and/or EPSPs ^a	13 of 14 (92.8%)	0 of 14 (0%) $P<0.0001^c$	0 of 14 (0%) $P<0.0001^c$, NS^d	0 of 13 (0%) $P<0.0001^c$, NS^d
Average level of intracellular Ca^{2+} , nM ^b	157.6±3.4 (n=243 neurons)	79.7±1.5 $P<0.001^c$ (n=181 neurons)	92.3±1.9 $P<0.001^c$, $P<0.01^d$ (n=160 neurons)	93.9±2.9 $P<0.001^c$, $P<0.01^d$ (n=132 neurons)
Neurons with spontaneous Ca^{2+} transients ^a	237 of 243 (97.5%)	16 of 181 (8.8%) $P<0.0001^c$	55 of 160 (34.3%) $P<0.0001^c$, $P<0.0001^d$	55 of 132 (41.7%) $P<0.0001^c$, $P<0.0001^d$
Frequency of spontaneous Ca^{2+} transients, transients/hr ^b	63.6±1.9 (n=237 neurons)	21.6±5.1 $P<0.001^c$ (n=16 neurons)	54.5±5.2 NS^c , $P<0.001^d$ (n=55 neurons)	42.9±4.2 $P<0.01^c$, $P<0.05^d$ (n=55 neurons)
Amplitude of glutamate (5 μM)- mediated Ca^{2+} increases, nM ^{b,g} (and the number of glutamate-responsive neurons)	157.0±9.2 (n=67 of 67 neurons)	68.7±6.7 $P<0.001^c$ (n=73 of 73 neurons)	116.3±9.2 $P<0.01^c$, $P<0.01^d$ (n=67 of 67 neurons)	122.1±11.6 $P<0.05^c$, $P<0.001^d$ (n=64 of 65 neurons)
Neurons with spontaneous action potentials ^a	8 of 14 (57.1%)	4 of 14 (28.6%) NS^c	9 of 14 (64.3%) $\text{NS}^{c,d}$	4 of 13 (30.8%) $\text{NS}^{c,d}$
Frequency of spontaneous action potentials, Hz ^b	1.5±0.5 (n=8 neurons)	1.8±0.6 (n=4 neurons)	1.6±0.2 $\text{NS}^{c,d}$ (n=9 neurons)	3.1±0.9 $\text{NS}^{c,d}$ (n=4 neurons)
Membrane potential, mV ^{b,h}	-63.3±2.1 (n=6 neurons)	-61.3±1.9 NS^c (n=10 neurons)	-59.8±2.1 $\text{NS}^{c,d}$ (n=5 neurons)	-61.0±1.2 $\text{NS}^{c,d}$ (n=9 neurons)
Input resistance, M Ω ^b	222.7±26.9 (n=14 neurons)	197.0±25.3 NS^c (n=14 neurons)	270.0±24.8 $\text{NS}^{c,d}$ (n=14 neurons)	267.1±44.9 $\text{NS}^{c,d}$ (n=13 neurons)

Measurements were taken without AP5/CNQX in the incubating medium in control cultures and in the presence of corresponding concentrations of AP5/CNQX in acute AP5/CNQX (100/10 μM), 1X (100/10 μM) and 2X (200/20 μM) cultures. Significance of difference was calculated using

^a Fisher's Exact Probability test and

^b ANOVA with post hoc Tukey test relative to the control

^c and acute AP5/CNQX

d cultures.

e Number of fields is analyzed.

f These tests were done in the presence of 50 μ M bicuculline.

g These tests were done in the presence of 2 μ M TTX.

h Average membrane potential was calculated for non-spiking neurons and neurons with the low action potential activity. In parentheses, the number of fields, neurons or percentage. NS, not significant. Table includes the data from both more-responsive and less-responsive batches.