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Development of Excitatory Synapses in Cultured Neurons Dissociated From the Cortices of Rat Embryos and Rat Pups at Birth

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We studied the development of excitatory synapses in cultured neurons dissociated from the cortices of rat embryos at the 18th day of gestation (E18) and rat pups at birth (P0). Between 7 and 14 days in vitro (DIV), large increases in the amplitudes and frequencies of the spontaneous excitatory postsynaptic currents (EPSCs) of both cultured E18 and P0 neurons were observed. The EPSCs of E18 neurons were mediated primarily by α-amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid (AMPA) receptors at 7 DIV and by both N-methyl-D-aspartate (NMDA) and AMPA receptors at 14 DIV. Consistently, immunostaining indicated significant increases in the proportion of the clusters of NR1, an NMDA receptor subunit, which were associated with the accumulation of synaptophysin, a presynaptic marker, in cultured E18 neurons between 7 and 14 DIV. The proportion of NR1 clusters residing in synaptic regions and the proportion of synapses that colocalized with NR1 clusters in 7-day-old P0 neurons were not different statistically from those found in 7-day-old E18 neurons. However, cultured P0 neurons at 7 DIV displayed clear EPSCs mediated by NMDA receptors. Our results suggest that the targeting of NMDA receptors to synaptic regions lag behind the synaptic clustering of AMPA receptors during the in vitro development of cultured rat E18 cortical neurons. The results further suggest that the cortical neurons at P0 differ from those at E19 in certain cellular properties; as a result, the currents mediated by the synaptic NMDA receptors in 7-day-old P0 neurons are larger than those mediated by the synaptic NMDA receptors in 7-day-old E18 neurons.

Key words: AMPA receptors; NMDA receptors; synaptogenesis; cortical neurons

Glutamate is a principle excitatory neurotransmitter in the mammalian central nervous system (CNS). In adult animals, fast central excitatory synaptic transmission is primarily mediated by the actions of glutamate on two types of ionotropic glutamate receptors, the α-amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, localized at the postsynaptic terminals (Hollmann and Heinemann, 1994; Kharazia and Weinberg, 1997; Conti and Weinberg, 1999). Recent studies have indicated that central glutamatergic synapses undergo alterations during postnatal development. In rat pups during the first 2 days after birth, the excitatory postsynaptic currents (EPSC) of the majority of CA1 neurons in the hippocampal slices are mediated by NMDA receptors alone. As development progresses, the percentages of CA1 neurons exhibiting EPSCs mediated by both AMPA and NMDA receptors gradually increase (Durand et al., 1996). Similar observations have also been made in developing frog brains and rat thalamocortical inputs (Wu et al., 1996; Issac et al., 1997). Anatomical studies have shown that the fibers of thalamus begin to invade the cerebral cortex and likely form synapses with the precursor principle neurons there in the late phase of embryonic development (for review, see Molnar and Blakemore, 1995). However, the receptors mediating the thalamocortical inputs during prenatal cortical development are still unclear.

The synaptogenesis of cultured neurons during in vitro development has also been investigated. Studies of developing hippocampal and spinal cord neurons in culture showed that AMPA receptors were recruited to synapses earlier than NMDA receptors (Rao et al., 1998; O'Brien et al., 1997). Other studies of cultured hippocampal and cortical neurons, on the other hand, indicated that both NMDA and AMPA receptors were present in syn-

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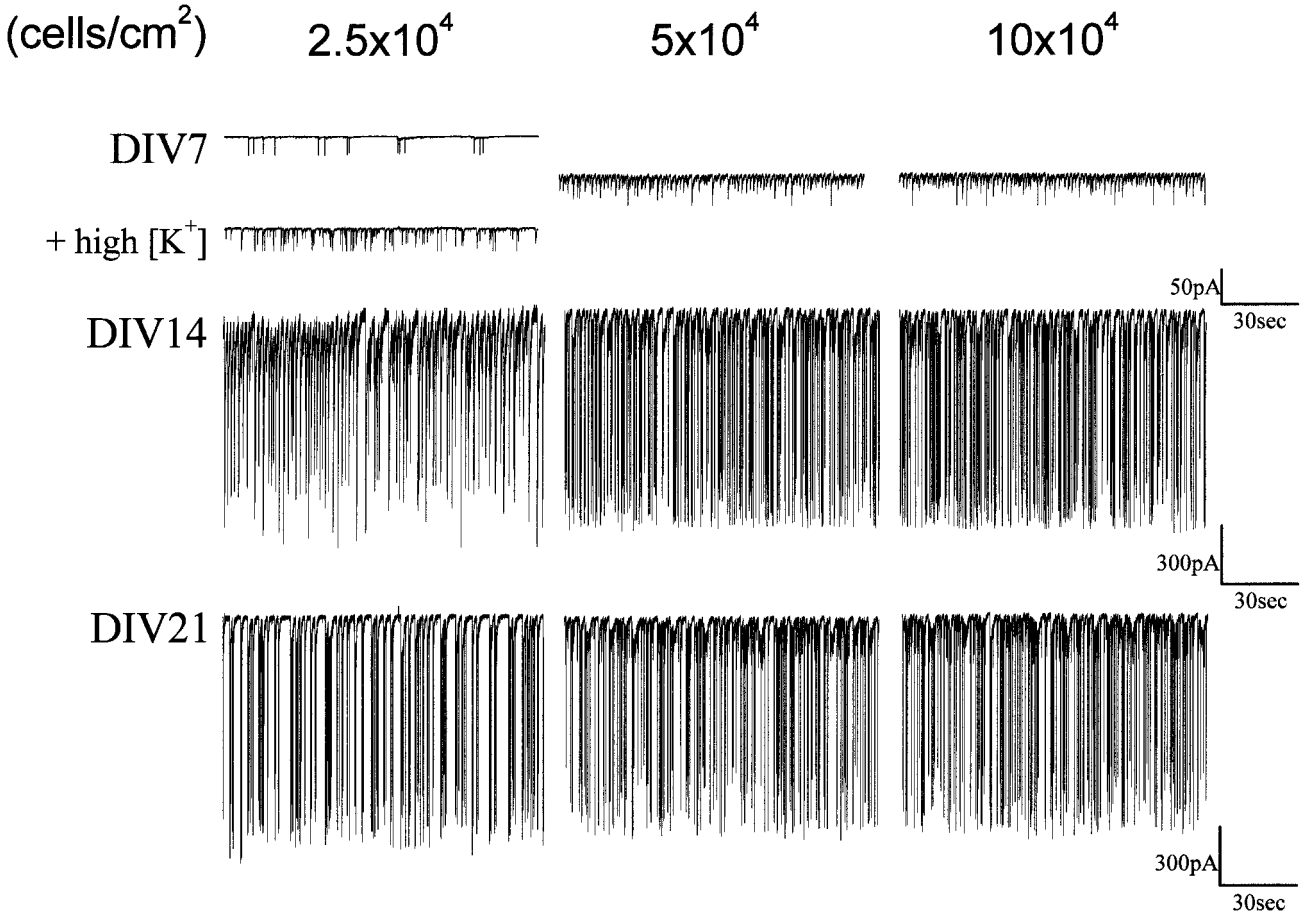

Fig. 1. Spontaneous excitatory postsynaptic currents (EPSCs) recorded from cultured embryonic day (E)18 cortical neurons at 7, 14, and 21 days in vitro (DIV) and at the cell densities of 2.5 (left column), 5 (middle column), and 10×10^4 (right column) cells/cm^2. The second trace from the top of the left column was recorded from a cultured neuron at 7 DIV in high potassium external solution containing 25 mM KCl.

apses within the first week in vitro (e.g., Jones and Baughman, 1991; Li et al., 1998; Liao et al., 1999; Gomperts et al., 2000; Pichard et al., 2000; Watt et al., 2000). The reasons behind the aforementioned controversial observations are yet unknown.

Here, we studied the development-dependent changes in the excitatory synapses of cultured neurons dissociated from the cortices of day 18 rat embryos (E18) and rat pups at birth (P0). The results indicated rapid synapse development of both E18 and P0 cortical neurons during the second week in culture. Both of our electrophysiological and immunostaining studies supported that the targeting of NMDA receptors to synaptic regions lagged behind the synaptic clustering of AMPA receptors during the in vitro development of E18 neurons. Our results also indicated that the currents mediated by the synaptic NMDA receptors in 7-day-old P0 neurons were larger than those mediated by the synaptic NMDA receptors in 7-day-old E18 neurons; as a result, the former neurons, but not the latter ones displayed clear NMDA receptor-mediated EPSCs. The implication of our present results to the types of glutamate receptors mediating the excitatory synaptic transmission in prenatal cerebral cortex is also discussed.

MATERIALS AND METHODS

Materials

Pregnant Sprague-Dawley rats were obtained from National Animal Laboratory, Taipei, Taiwan. HEPES, glucose, poly-D-lysine, cysteine, Triton X-100, bovine serum albumin, papain, cytosine arabinoside (ARC), and DNaseI were purchased from Sigma (St. Louis, MO). Strychinine, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), D-APV (D-2-amino-5-phosphovalerate), tetrodotoxin (TTX), and bicuculline were obtained from Research Biochemical International (Natick, MA). Dulbecco's modified Eagle medium (DMEM) powder was obtained from Gibco (Grand Island, NY). Horse and fetal calf sera were purchased from Biological Industries (Haemek, Israel). Mouse antibodies against synaptophysin were purchased from Boehringh-Mannheim (Indianapolis, IN), and rabbit an-

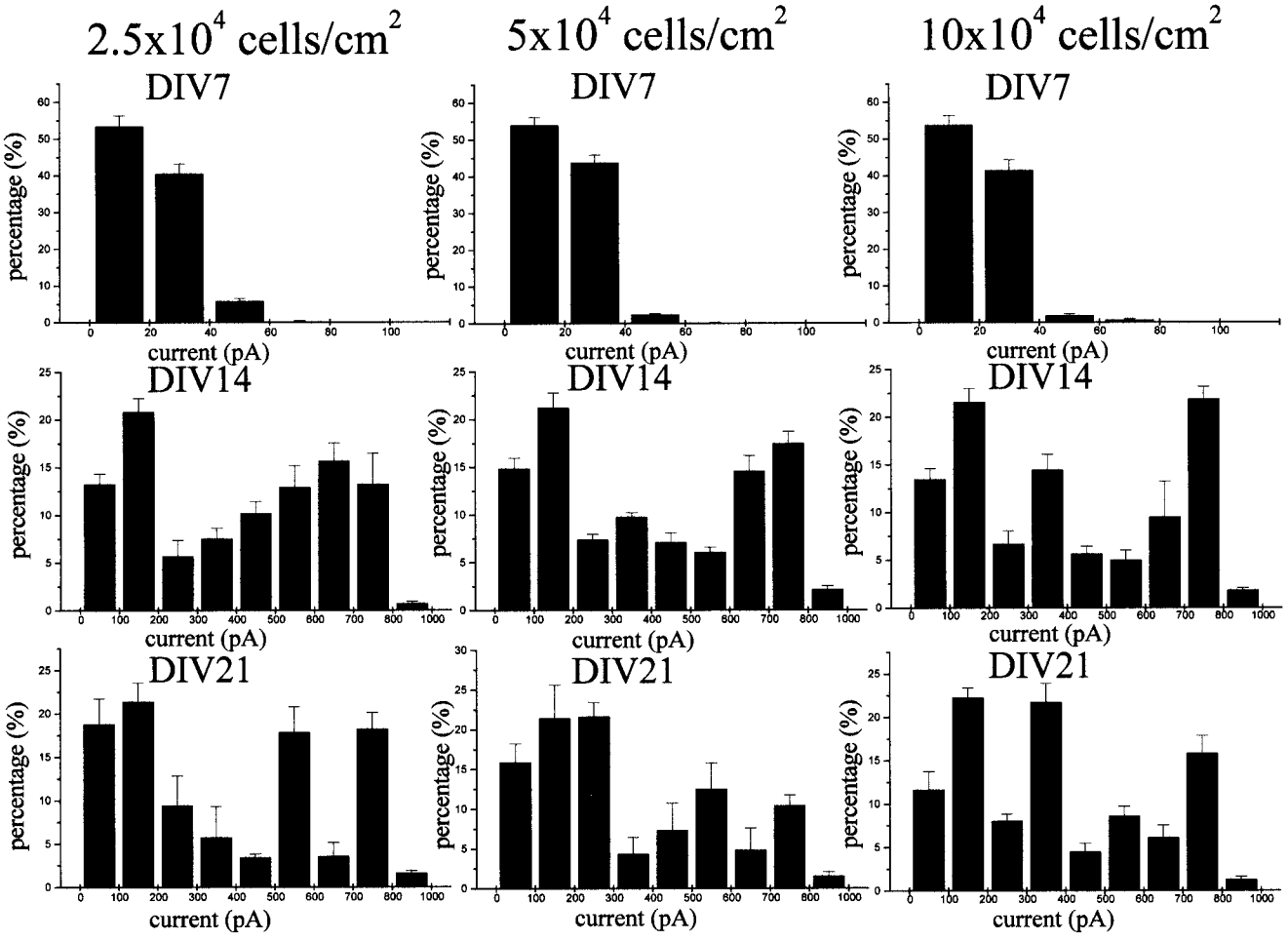

Fig. 2. The distribution of the EPSC amplitudes during a period of 1 min in cultured E18 cortical neurons at 7, 14, and 21 DIV and at the cell densities of 2.5, 5, and 10×10^4 cells/cm^2. Error bars represent standard error of mean (n = 4 to 12).

tisera against AMPA receptor subunit GluR1 and NMDA receptor subunit NR1 were obtained from Chemico Company (Temecula, CA). Texas-Red-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). Other reagents were obtained from Merck-Schduardt.

Cortical Cell Culture

Neuron-enriched primary cortical cultures were prepared from Sprague-Dawley rats as described elsewhere (Chou, 1998). In brief, cortices were dissected from rat embryos (18th day of gestation, E18) or rat pups on the day of birth (P0) and treated with papain (10 units/ml). Afterwards, the dissociated cells were washed and suspended in DMEM supplemented with 10% horse serum. Cells, at the density of 2.5, 5, or 10×10^4 cells/cm^2, were then plated onto microscopic coverglasses (18 mm in diameter, Assistent, Germany), precoated with poly-D-lysine and placed in the wells of 24-well culture plates, and incubated at 37°C in a humidified incubator with 5% CO_2/95% air for 3 days. Thereafter, cells were treated with 5 μM ARC for 1 day. The culture medium was subsequently replaced with 10% horse serum/DMEM every 5 days.

Electrophysiology

Conventional patch-clamp techniques (Tyan et al., 1997) were used. The resistance of borosilicate electrodes when filled with the internal solution (in mM: K+-gluconate 140, EGTA 2, HEPES 10, glucose 10, ATP-Na_2 4, pH 7.4) was 5–8 MΩ. Cells were superfused by the external solution (in mM: NaCl 145, KCl 3, $CaCl_2$ 2, Hepes 10, at pH 7.4) containing 30 μM strychinine and 20 μM bicuculline. In some cases, high potassium external solution, containing 123 mM NaCl, 25 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 30 μM strychinine and 20 μM bicuculline at pH 7.4, was used to evoke more EPSC. The membrane potential of neurons was clamped at −60 mV during recording. Membrane currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA) in the whole-cell configuration, digitized, stored and analyzed by an IBM computer with the software pClamp6 (Axon). Currents recorded with an Axopatch 200 amplifier were also stored on the magnetic tape (Panasonic video recorder) and

TABLE I. Effects of CNQX and D-APV on the Frequencies of the EPSCs of Cultured E18 Cortical Neurons★

	Before (spikes/sec)	CNQX (spikes/sec)	CNQX + APV (spikes/sec)
7 DIV			
2.5×10^4 (cells/cm^2)[a]	0.51 ± 0.03 (12)	0 (11/12), 0.24 (1/12)	0 (12)
5×10^4 (cells/cm^2)	0.84 ± 0.04 (8)	0 (8)	—
10×10^4 (cells/cm^2)	0.93 ± 0.03 (6)	0 (4/6), 0.35 (1/6), 0.50 (1/6)	0 (6)
14 DIV			
2.5×10^4 (cells/cm^2)	1.38 ± 0.05 (8)	1.02 ± 0.07 (8)	0 (8)
5×10^4 (cells/cm^2)	1.43 ± 0.05 (8)	1.11 ± 0.08 (8)	0 (8)
10×10^4 (cells/cm^2)	1.53 ± 0.07 (5)	1.28 ± 0.07 (5)	0 (5)
21 DIV			
2.5×10^4 (cells/cm^2)	1.25 ± 0.07 (8)	0.96 ± 0.09 (8)	0 (8)
5×10^4 (cells/cm^2)	1.56 ± 0.06 (4)	1.30 ± 0.06 (4)	0 (4)
10×10^4 (cells/cm^2)	1.61 ± 0.06 (4)	1.37 ± 0.12 (4)	0 (4)

★Cultured embryonic day (E)18 cortical neurons at 7, 14, and 21 days in vitro (DIV) and at different cell densities were recorded in the whole-cell recording configuration with a holding membrane potential of −60 mV and in Mg^{2+}-free external solution. Data are mean ± standard error of mean of the frequencies of excitatory postsynaptic currents (EPSCs) of neurons during a period of 1 min (n, in parenthesis) before, during the application of 10 μM CNQX, and during the coapplication of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM D-2-amino-5-phosphovalerate (D-APV).

[a]Recorded in high potassium external solution containing 25 mM KCl.

then analyzed by the IMB computer. Inward currents larger than twice of the background noise (~5 pA) were taken as EPSCs for further analysis.

Fluorescence Immunostaining

For labeling the AMPA receptor subunit GluR1 and synaptophysin of cultured cortical neurons, cells were fixed by 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and then permeabilized in 0.25% Triton X-100 for 5 min. After incubating cells in normal goat serum (10% in PBS) at 37°C for 1 hr, cells were incubated with a mixture containing mouse anti-synaptophysin antibody and rabbit anti-GluR1 antiserum at 4°C overnight. Cells were then incubated sequentially with Texas Red-conjugated anti-mouse secondary antibody at 37°C for 45 min and with FITC-conjugated anti-rabbit secondary antibody at 37°C for 45 min. For immunostaining the NMDA receptor subunit NR1 and synaptophysin of cultured cortical neurons, cells were fixed and permeabilized with methanol at −20°C for 15 min. After incubating with normal goat serum (10% in PBS) at 37°C for 1 hr, cells were incubated with mouse anti-synaptophysin antibody at 37°C for 2 hr and then with rabbit anti-NR1 antiserum at 37°C for 2 hr. Subsequently, cells were incubated with Texas Red-conjugated anti-mouse secondary antibody at 37°C for 45 min and then with FITC-conjugated anti-rabbit secondary antibody at 37°C for 45 min. Cells were sealed in Prolong Antifade (Molecular Probes). An epifluorescence microscope (Nikon, Optiphot-2) was used in this study. A 100× oil lens (N.A. 1.30, Plan Fluor 100, Nikon, Japan) was used in NR1/synaptophysin-staining experiments, and a 40× oil lens (N.A. 1.30, Plan Fluor 40, Nikon, Japan) was used in GluR1/synaptophysin-staining experiments. Photomicrographs were prepared from digital scans of slides with a Nikon slide scanner (Cool Scan III). The contrast and brightness were adjusted digitally by Photoshop software (Adobe Systems Inc., San Jose, CA).

RESULTS

Spontaneous EPSCs of cultured E18 cortical neurons at the cell density of 5×10^4 cells/cm^2 were recorded at 7, 14, and 21 days in vitro (DIV; middle column of Fig. 1). Large increases in the amplitudes (middle columns of Figs. 1 and 2) and frequencies (Table I) of the EPSCs were observed between 7 and 14 DIV. At 7 DIV, the amplitudes of EPSCs were less than 100 pA, and at 14 DIV the amplitudes of EPSCs distributed between 0 and 1,000 pA. The average EPSC frequency of the neurons at 14 DIV was nearly twice of that of the neurons at 7 DIV. These observations suggested the making of new synapses and increases in the number of glutamate receptors of cultured E18 neurons during the second week in vitro. The frequencies and amplitudes of EPSCs recorded from neurons at 14 and 21 DIV were similar (Table I and the middle columns of Figs. 1 and 2), suggesting the maturation of synaptic connections of cultured E18 neurons after the second week in vitro.

Antagonists for non-NMDA and NMDA receptors, CNQX and D-APV, respectively, were employed here to examine the types of glutamate receptors that mediated the EPSCs of cultured E18 neurons at 7, 14, and 21 DIV. At 7 DIV, the EPSCs were completely inhibited by the application of CNQX (10 μM) alone and by the coapplication of CNQX (10 μM) and D-APV (50 μM), but not significantly affected by the application of D-APV (50 μM) alone (left and middle columns of Fig. 3 and Table I). The EPSCs recorded from neurons at 14 and 21 DIV were partially blocked by the application of CNQX (10 μM) alone and completely blocked by the coapplication of CNQX (10 μM) and D-APV (50 μM; Table I and the results from the experiments of 14 DIV neurons were shown as the right column of Fig. 3). Table I summarizes the frequencies of the EPSCs recorded from cultured

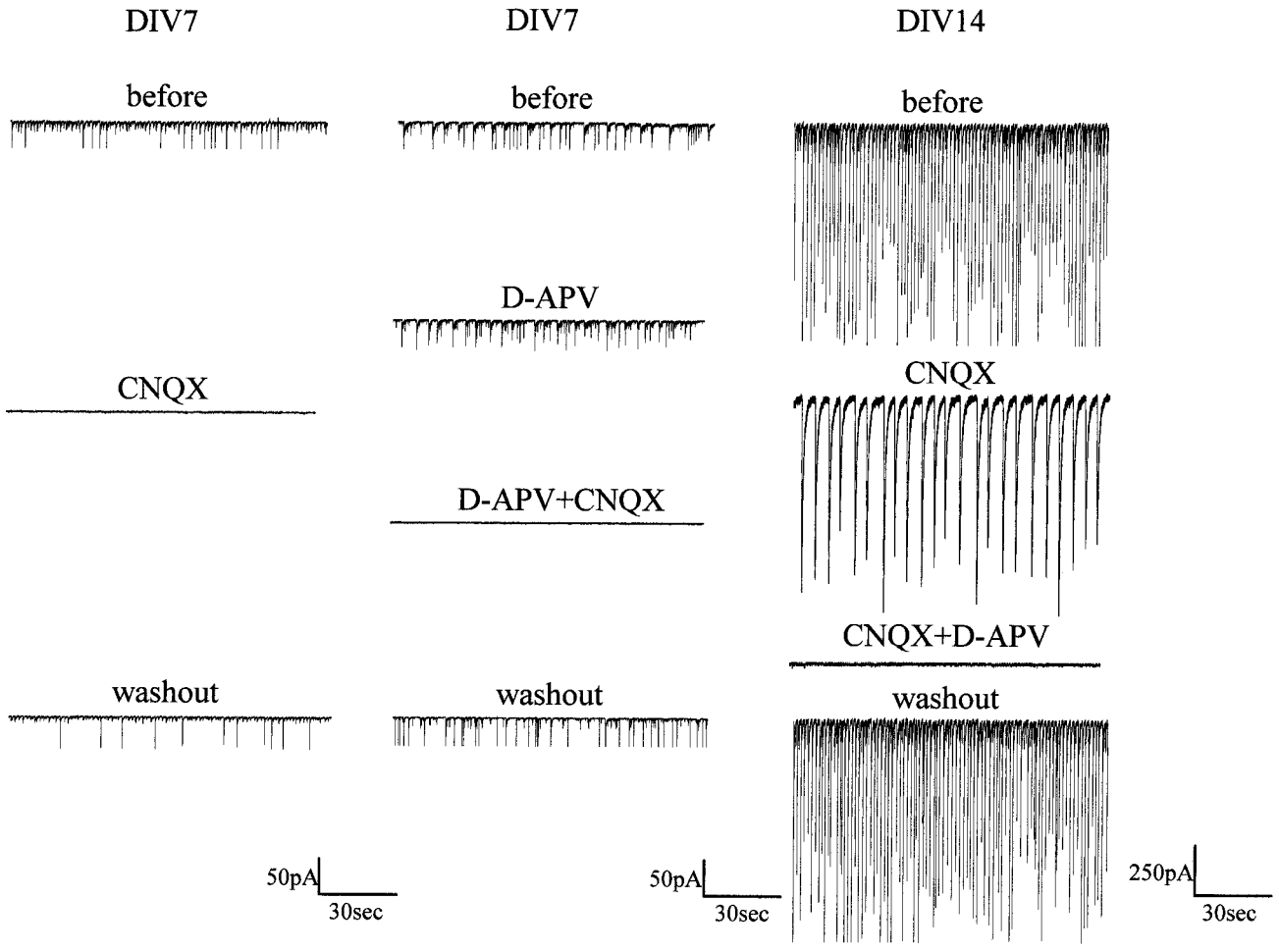

Fig. 3. Pharmacological characterization of the EPSCs of cultured E18 cortical neurons at 7 and 14 DIV. **Left column:** The EPSCs of a cultured neuron at 7 DIV (top trace) was completely blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM; middle trace). **Middle column:** The EPSCs of cultured neurons at 7 DIV (top trace) was not significantly affected by D-2-amino-5-phosphovalerate (D-APV; 50 μM; second trace from the top) and completely blocked by CNQX (10 μM) plus D-APV (50 μM; third trace from the top). **Right column:** The EPSCs of cultured neuron at 14 DIV (top trace) were partially blocked by CNQX (10 μM; middle trace) and completely blocked by CNQX (10 μM) plus D-APV (50 μM).

cortical neurons at 7, 14, and 21 DIV and in the presence of different antagonists. These results indicated that at 7 DIV, the EPSCs of cultured E18 cortical neurons were exclusively mediated by non-NMDA receptors, while at and after 14 DIV the EPSCs of cultured neurons were mediated by both NMDA and non-NMDA receptors.

Because the variation in cell densities was reported to affect the in vitro synaptogenesis of rat hippocampal and hypothalamic neurons (Van Den Pol et al., 1998), we then examined the development of excitatory synapses of cortical neurons maintained at different cell densities. The EPSCs of cultured E18 cortical neurons at the densities of 2.5 and $10 \times 10^4/cm^2$ were also recorded at 7, 14, and 21 DIV (left and right columns of Figs.1 and 2). The EPSC frequencies of E18 cortical neurons at the cell density of 2.5×10^4 cells/cm^2 were very low (zero in many cases). To evoke more EPSCs, these neurons were recorded in a high potassium external solution containing 25 mM KCl (an example was given as the second trace from the top of the left column of Fig. 1). Significant increases in the frequencies and amplitudes of the EPSCs of neurons, maintained at 2.5 or 10×10^4 cells/cm^2, were observed between 7 and 14 DIV, and fewer changes in the frequencies and amplitudes of the EPSCs were observed between 14 and 21 DIV (Figs. 1 and 2 and Table I). The EPSCs recorded from most of the neurons (11 out of 12 neurons at 2.5×10^4cells/cm^2 and four out of six neurons at 10×10^4cells/cm^2) at 7 DIV were completely blocked by the application of CNQX (10 μM) alone (Table I). The EPSCs recorded from cultured neurons at 14 and 21 DIV were only partially blocked by the application of CNQX (10 μM) alone and completely blocked by the coapplication of CNQX (10 μM) and D-APV (50 μM; Table I). These results indicated that the EPSCs of most of 7-day-

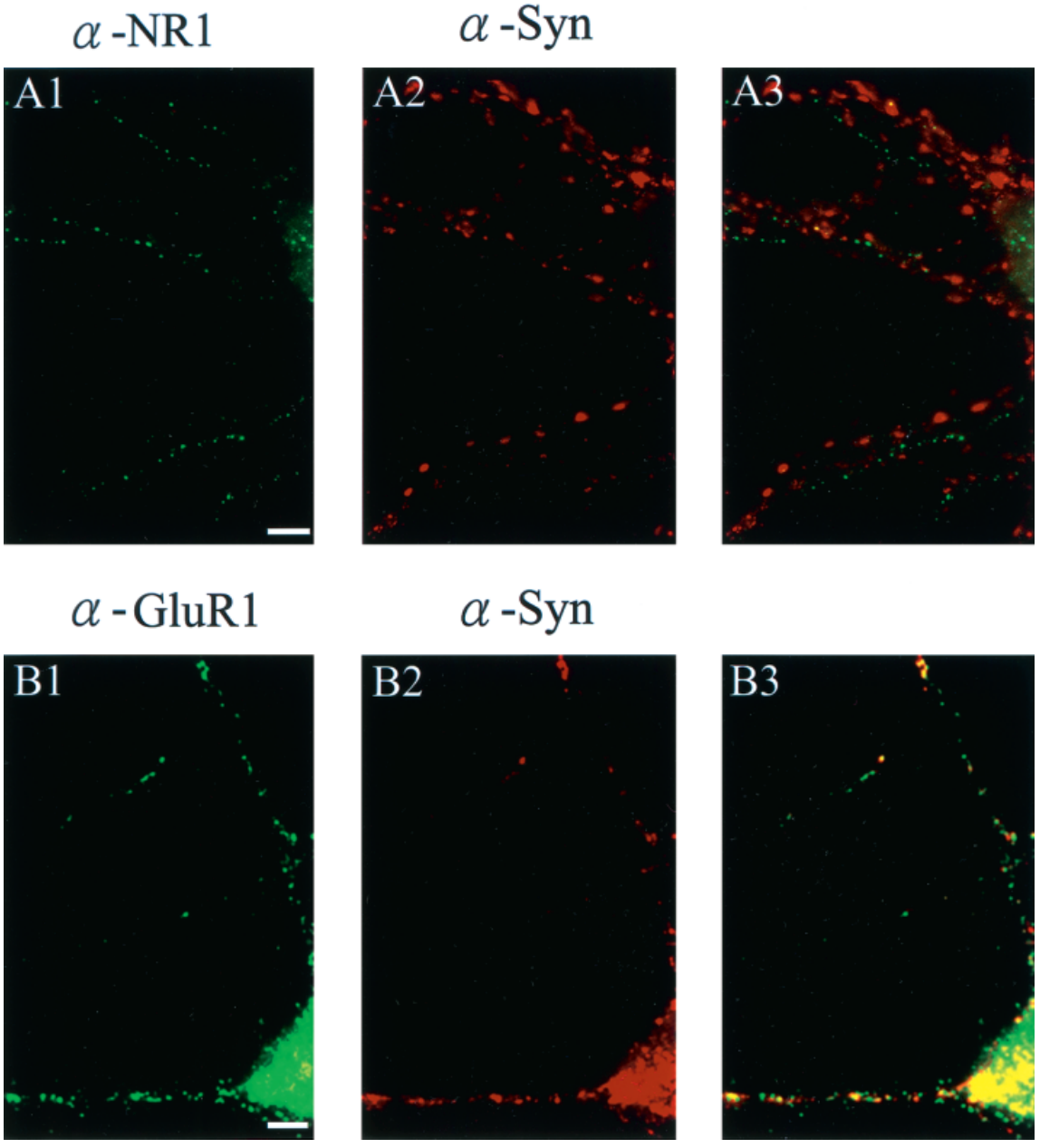

Fig. 4. Synaptic localization of AMPA receptor subunit GluR1 and NMDA receptor subunit NR1 in cultured E18 cortical neurons at 7 DIV. Fixed and permeabilized neurons were double-labeled with the antibodies to NR1 (**A1**) and synaptophysin (**A2**) or with the antibodies to GluR1 (**B1**) and synaptophysin (**B2**). Superimposed figures (**A3** and **B3**) indicate that the majority of NR1 clusters do not colocalize with synaptophysin accumulation (A3), while the majority of GluR1 clusters colocalize with synaptophysin accumulation (B3). Scale bars in A1 and B1 = 10 and 5 μm, respectively.

old cultured E18 cortical neurons were mediated by AMPA receptors alone regardless of the different cell densities (between 2.5 and 10 × 10^4cells/cm^2) at which they were maintained.

Fluorescence immunostaining was used here to investigate the expression of the AMPA receptor subunit GluR1 and NMDA receptor subunit NR1 at synapses in cultured cortical neurons. GluR1 or NR1 in neurons was labeled by FITC, and synaptophysin in the same neurons was tagged with Texas-Red. Clusters of GluR1 subunits were found on the processes of cultured neurons (Fig. 4B1–B3), and 63.4 ± 4.1% (n = 7) and 82.8 ± 9.4% (n = 7) of GluR1 clusters were associated with presynaptic synaptophysin accumulation in cultured E18 neurons at 7 and 14 DIV, respectively. Discrete clusters of NR1 subunits were also found along the processes of cultured E18

TABLE II. Percentage of NR1 Clusters Associated With Presynaptic Synaptophysin Accumulation⋆

	DIV	Cell density ($\times 10^4$ cells/cm^2)	Percentages of NR1 clusters associated with synaptophysin-positive spots
E18 neurons	7	2.5	8.1 ± 11.0% (n = 14, t = 300)[a]
		5	7.6 ± 9.3% (n = 14, t = 253)[b]
		10	3.7 ± 6.4% (n = 16, t = 284)
	14	2.5	21.5 ± 9.4% (n = 13, t = 625)
		5	20.5 ± 7.7% (n = 8, t = 208)
P0 neurons	7	5	12.9 ± 8.7% (n = 16, t = 388)

⋆Quantification of the proportions of NR1 clusters associated with presynaptic synaptophysin accumulation in cultured embryonic day E18 neurons at 7 and 14 days in vitro (DIV) and in cultured postsynaptic day P0 neurons at 7 DIV. Neurons were double-labeled with the antibodies to NR1 and synaptophysin as described in Materials and Methods. Discrete NR1 clusters on the processes extending from pyramidal-shaped cell bodies were taken into analyses, and areas were excluded from quantification where identification of processes was ambiguous. Data were means ± S.D. of the percentages of NR1 clusters colocalized with synaptophysin accumulation with numbers of cells (n) and total of numbers of NR1 clusters (t) shown in parentheses.

[a]Statistically significant differences from the 14-day-old neurons at 2.5×10^4 cells/cm^2 ($P < 0.01$; Student's *t*-test).

[b]Statistically significant differences from the 14-day-old neurons at 5×10^4 cells/cm^2 ($P < 0.01$; Student's *t*-test).

neurons. However, only 3–9% of NR1 clusters was associated with presynaptic synaptophysin accumulation (Table II and Fig. 4A1–A3), and the remaining NR1 clusters resided in extrasynaptic regions. At 14 DIV, the percentage of NR1 clusters associated with synaptophysin accumulation increased significantly to 20–22% (Table II). The increase in the proportions of NR1 clusters colocalizing with synaptophysin accumulation was consistent with our electrophysiological experiments in which NMDA receptor-mediated EPSCs were detected in 14-day-old neurons, but not in 7-day-old neurons. In addition, because no NMDA receptor-mediated EPSC was detected in most 9-day-old E18 neurons, the currents mediated by those NMDA receptor clusters that colocalized with ~4–9% of the synapses in these neurons were most likely very small and below our detection limit, as suggested by Robert et al. (2000).

Cortical neurons were also dissociated from rat pups at birth (P0) and maintained under in vitro conditions. Similar to those found in cultured E18 neurons, the amplitudes of the EPSCs of cultured P0 neurons increased from less than 100 pA at 7 DIV to several hundred pA at 14 DIV (Fig. 5). The average EPSC frequency of cultured P0 neurons at 14 DIV was nearly twice of that of the neurons at 7 DIV, 1.83 ± 0.10 (n= 3) and 0.93 ± 0.02 (n= 3), respectively. However, unlike 7-day-old E18 cortical neurons whose EPSCs were completely blocked by CNQX (Fig. 3, middle trace of the left column), the EPSCs recorded from P0 neurons at 7 and 14 DIV were only partially inhibited by the application of CNQX (10 μM) alone and completely blocked by the coapplication of CNQX (10 μM) and D-APV (50 μM; Fig. 6). The frequencies of spontaneous EPSCs of 7-day-old P0 neurons in the absence and presence of 10 μM CNQX were 0.93 ± 0.02 (n= 3) and 0.73 ± 0.06 (n= 3), respectively. The frequencies of spontaneous EPSCs of 14-day-old P0 neurons in the absence and presence of 10 μM CNQX were 1.83 ± 0.10 (n= 3) and 1.04 ± 0.15 (n= 3), respectively. These observations indicated that both AMPA and NMDA receptors mediated the EPSCs of cultured neurons at 7 DIV as well as at 14 DIV. Fluorescence immunostaining, however, indicated only 12.0 ± 8.7% of NR1 clusters on the processes of 7-day-old P0 neurons colocalized with synaptophysin accumulation (Table II). This number was not statistically different from the proportion of NR1 clusters that were associated with synaptophysin accumulation found in 7-day-old E18 neurons at the same cell density (by Student's *t*-test). The proportions of synaptophysin-positive spots that colocalized with NR1 clusters on the processes of 7-day-old E18 and P0 neurons were not different statistically, 7.9 ± 10.2% (n= 14) and 12.8 ± 8.3% (n= 16), respectively. Because NMDA receptor-mediated EPSCs could be detected in 9-day-old P0 neurons but not in 9-day-old E18 neurons, the currents mediated by the NMDA receptor clusters residing at synapses in the former neurons were likely larger than those mediated by the NMDA receptor clusters residing at synapses in the latter neurons.

DISCUSSION

In this study, we found that the EPSCs of cultured E18 cortical neurons were mediated almost exclusively by AMPA receptors at 7 DIV and by both AMPA and NMDA receptors at 14 and 21 DIV. Consistently, fluorescence immunostaining revealed significant increases in the percentage of NR1 clusters associated with synaptophysin accumulation of cultured E18 neurons between 7 and 14 DIV. These observations indicate that the recruitment of NMDA receptors to synaptic regions lags behind the synaptic targeting of AMPA receptors during the in vitro development of rat E18 cortical neurons.

We also recorded the EPSCs of cultured P0 neurons at 7 and 14 DIV. A comparison of the frequencies and amplitudes of EPSCs showed that the progression of the formation of excitatory synapses in E18 and P0 neurons during the first 2 weeks in culture was very similar. Furthermore, the proportions of NR1 clusters residing in synaptic regions and the proportions of synapses associated with NR1 clusters in 7-day-old E18 and 7-day-old P0 neurons were similar. These similarities thus suggest that E18 and P0 cortical neurons share common machinery regulating the growth of processes and formation of excitatory synapses during in vitro development. However, unlike the E18 neurons at 7 DIV with the EPSCs being mediated nearly exclusively by AMPA receptors, we could detect clear EPSCs mediated by both AMPA and NMDA receptors in P0 neurons at 7 DIV. This difference further suggests that the currents mediated by the NMDA receptor clusters residing in the synaptic regions in 7-day-old P0

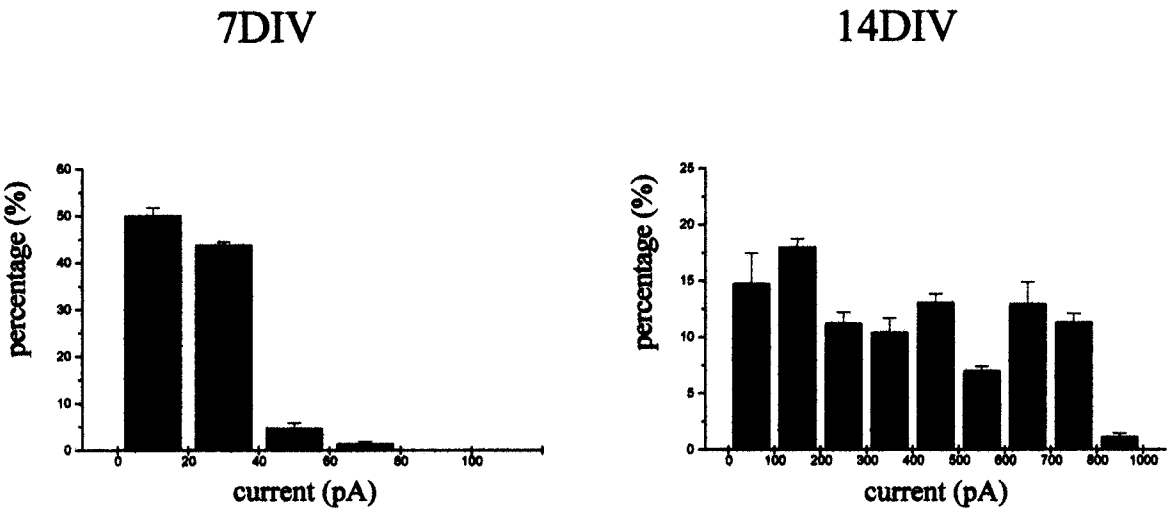

Fig. 5. The distribution of the EPSC amplitudes during a period of 1 min in cultured P0 cortical neurons at 7 and 14 DIV. Error bars represent standard error of mean (n = 3).

neurons are larger than the currents mediated by NMDA receptor clusters residing in the synaptic regions in 7-day-old E18 neurons. This latter difference in currents may be due to distinct subunit compositions or various modifications of synaptic NMDA receptors or different synaptic efficacies in cultured E18 and P0 neurons at 7 DIV (Monyer et al., 1994; Li et al., 1998; reviews of Hollmann and Heinemann, 1994; McBain and Mayer, 1994).

Our results of cultured P0 cortical neurons are consistent with those reported by Jones and Baughman (1991) and Li et al. (1998). Jones and Baughman (1991) demonstrated the colocalization of AMPA and NMDA receptors in cultured P4–P6 cortical neurons at 7 DIV; Li et al. (1998) showed the appearance of NMDA receptors in synapses of cultured P1 cortical neurons at 6 DIV. Studies of cultured hippocampal neurons dissociated from postnatal rats also showed that both AMPA and NMDA receptors mediated EPSCs during the first week in vitro (e.g., Pichard et al, 2000; Watt et al., 2000; Gomperts et al., 2000). Our finding of the delayed recruitment of NMDA receptors to synapses in cultured E18 cortical neurons is also found by Rao et al. (1998) and O'Brien et al. (1997) in their studies of the development of excitatory synapses in cultured neurons dissociated from embryonic hippocampus and spinal cord, respectively. However, our results of cultured E18 neurons are inconsistent with that reported by Liao et al. (1999) who showed that pure NMDA receptor synapses, which lacked AMPA receptors, accounted for most of the synapses in 7-day-old cultured E18 hippocampal neurons. The reasons behind this difference is presently unclear; it is probably due to the differences in culturing conditions, in the sensitivities of the immunostaining methods, or in the sensitivities between the immunostaining and electrophysiological methods used by Liao et al. (1999) and us.

Recent studies have indicated that AMPA and NMDA receptors are recruited to synapses by different mechanisms (for reviews, see Nusser, 2000; Lee and Sheng, 2000). The AMPA receptors, at least a portion of these receptors, appear to undergo redistribution into and out of synapses continuously, and synaptic activity and the activities of some intracellular signaling molecules have been proposed to regulate the redistribution of AMPA receptors (Lin and Sheng, 1998; Turrigiano, 2000; Man et al., 2000; Lin et al., 2000; Beattie et al., 2000; Lu et al., 2001). Compared to AMPA receptors, the attachment of NMDA receptors to synaptic junctions appears to be stronger (Chang et al., 1996). Nevertheless, the subunit composition and expression level of NMDA receptors are also regulated by activity (Quinlan et al., 1999; Shi et al., 2000; Philpot et al., 2001). More work is required to test whether any one of the aforementioned mechanisms or a new mechanism may account for the observed delayed recruitment of NMDA receptors to synapses during the in vitro development of E18 cortical neurons.

Starting from E15, the precursor principle neurons of developing cerebral cortex migrate from the ventricular zone into the middle of preplate and form a dense layer there, called cortical plate (Molnar and Blakemore, 1995). Starting from E18, thalamus input begins to invade into the cortical plate. As a result, the principal neurons in the cortical plate receive inputs from developing thalamus before birth, which takes place 3 days later (P0). Information regarding the excitatory synaptic transmission among neurons in embryonic brains is limited. By extracellular recording from intact hippocampal formation preparations isolated from E19 rat embryos, Diabira et al. (1999) found that the field excitatory postsynaptic potentials were primarily mediated by the AMPA receptors, with the NMDA receptors contributing to only ~10% of the synaptic response. Our present study indicates that the excitatory synaptic transmission between cultured E18 cortical neurons is almost exclusively mediated by AMPA re-

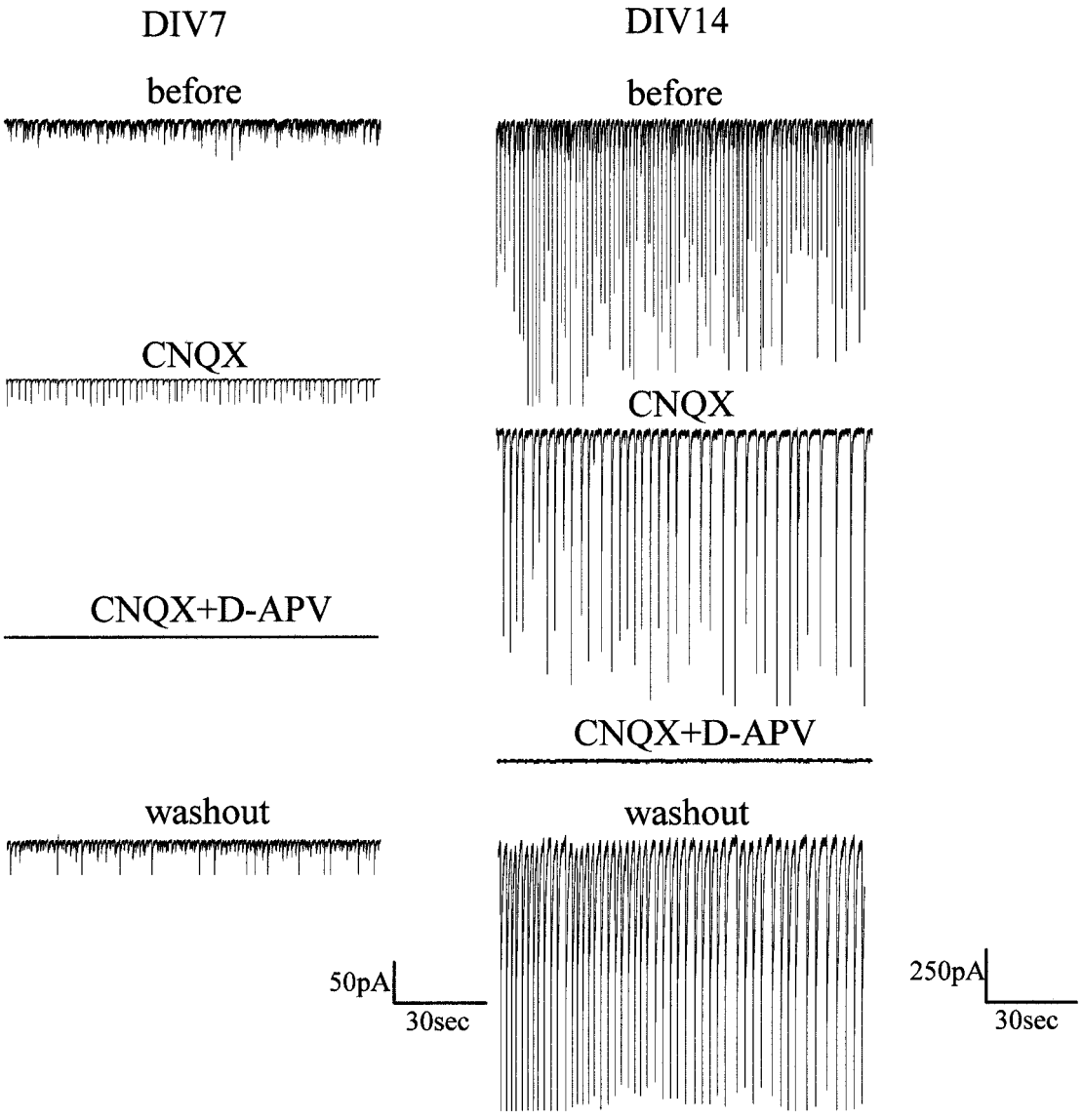

Fig. 6. Pharmacological characterization of the EPSCs of cultured P0 cortical neurons at 7 and 14 DIV. **Left column:** The EPSCs of a cultured neuron at 7 DIV (top trace) was partially blocked by CNQX (10 μM; second trace from the top) and completely blocked by CNQX (10 μM) and D-APV (50 μM; third trace from the top). **Right column:** The EPSCs of cultured neurons at 14 DIV (top trace) was partially blocked by CNQX (10 μM; second trace from the top) and completely blocked by CNQX (10 μM) plus D-APV (50 μM; third trace from the top).

ceptor initially and by both NMDA and AMPA receptors as neurons become more developed. Together, these results raise a possibility that a similar change in the types of glutamate receptors mediating excitatory synaptic transmission also takes place during embryonic development. Further investigation is required to test this possibility.

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