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CRITICAL PERIOD FOR ACTIVITY-DEPENDENT ELIMINATION OF CORTICOSPINAL SYNAPSES *IN VITRO*

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Abstract—There is no *in vitro* model of the critical periods for developmental plasticity, the time windows of plastic changes during development, which may hinder in-depth mechanistic analysis. We have shown previously that the corticospinal tract with synaptic connections can be reconstructed in *in vitro* co-cultures using slices of the sensorimotor cortex and spinal cord of the rat. In our *in vitro* system, corticospinal synapses form widely over spinal gray matter during early development, after which those on the ventral side are eliminated in an activity and *N*-methyl-D-aspartate (NMDA)-dependent manner. A detailed quantitative analysis of the time course of sensitivity to an NMDA blocker was made with this system. Synapse distribution was evaluated by recording field excitatory post-synaptic potentials evoked by deep cortical layer stimulation. Corticospinal axon terminal distribution was examined by anterograde labeling with biocytin. We showed that the D-2-amino-5-phosphonovaleric acid (APV) effect is irreversible for at least the length of culture. When APV was removed from the medium before 6 days *in vitro*(DIV) or after 11 DIV, elimination of ventral synapses was not blocked. APV sensitivity showed a clearly defined time window. A 6–11 DIV application was necessary and sufficient for the full, irreversible block of synapse elimination. From 6–11 DIV, APV sensitivity seems to decrease gradually but not linearly. This system provides an *in vitro* model of critical periods for developmental plasticity of central synapses which up to now has not been available. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: spinal cord, motor cortex, slice culture, development, plasticity.

Immature brain has surprisingly high modifiability that often is lost with maturation. In early development of the brain, in particular, there are relatively narrow time windows, called critical periods, during which extrinsic events of short duration have lifelong influence on the neuronal circuits that underlie an individual's responses and behavior. These extrinsic events, however, do not show profound effects before or after a given critical period. Critical periods have been reported in various parts of the CNS, including the visual (Hubel and Wiesel, 1970; Malach et al., 1984), somatosensory (Jeanmonod et al., 1981), and auditory systems (Knudsen et al., 1991), and the cerebellum (Kakizawa et al., 2000; see references O'Leary et al., 1994; Berardi et al., 2000; Hensch, 2004 for review). The mechanism that controls these critical periods may be a complicated composite one. *In vitro* analysis of these developmental events should be of great help in unraveling this complexity and clarifying its aspects, but no *in vitro* model of a critical period in the CNS exists.

Using sensorimotor cortex and spinal cord slice co-cultures, we established an *in vitro* corticospinal synapses preparation (Takuma et al., 2002). Our preparation reliably records field excitatory post-synaptic potentials (fEPSPs; the extracellular manifestation of excitatory post-synaptic potentials) from the spinal cord in response to cortical stimulation (Takuma et al., 2002). Systematic recordings of fEPSPs allowed quantitative evaluation of the formation of corticospinal synapses and their spatial distribution (Ohno et al., 2004). Retrograde labeling by 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), or horseradish peroxidase (Takuma et al., 2002), as well as fluorescent latex microspheres (Beads; Ohno et al., 2004) of axons extending from the cortical to spinal tissue showed they were corticospinal neurons. Most DiI-labeled cells were large pyramidal-like ones distributed in a specific pattern in the deep cortical region (Takuma et al., 2002). Anterograde labeling of these cells with biocytin placed in the deep cortical layer showed the spatial distribution of corticospinal axons. The terminal distribution of the corticospinal fibers coincided well with the termination area of the corticospinal tract *in vivo*; the distribution of fEPSPs and closely paralleled the anterogradely labeled axon terminals (Ohno et al., 2004).

In the later developmental stage of the nervous system, exuberant axons and synapses are eliminated, often activity dependently (Changeux and Danchin, 1976; Purves and Lichtmen, 1980; Constantine-Paton et al., 1990; Katz and Shatz, 1996; Lohof et al., 1996). Corticospinal synapses in our system also were formed throughout the spinal gray matter at 7 days *in vitro*(DIV), whereas synapses in the ventral area were eliminated up to 14 DIV. This synapse decrease primarily was due to axon branch elimination and was activity dependent (Ohno et al., 2004). Synapse elimination was blocked when 50 μM 2-amino-5-phosphonovaleric acid (APV), an NMDA receptor antagonist, was present from 0 to 14 DIV. We here examine whether there is a critically sensitive period for this APV blocking effect.

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Abbreviations: APV, D-2-amino-5-phosphonovaleric acid; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DIV, days *in vitro*; fEPSPs, field excitatory post-synaptic potentials; PB, phosphate buffer.

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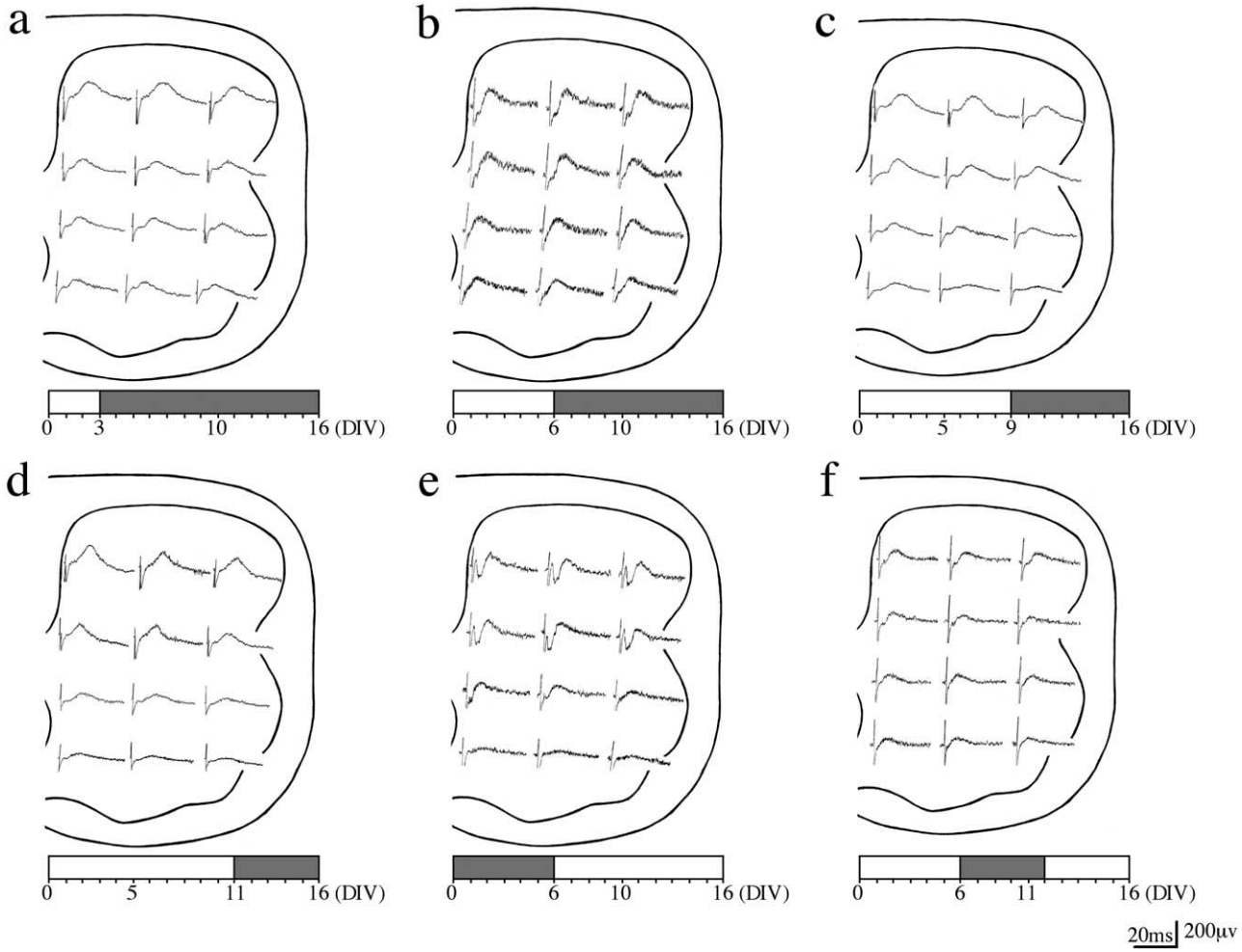

Fig. 1. (a–f) Spatial distribution of fEPSPs at 16 DIV for the APV application periods shown below each panel. fEPSPs were recorded along a 100 μm-interval lattice in the spinal gray matter. Even-numbered rows in both the dorso-ventral and medio-lateral lanes have been omitted to show each wave more clearly. Culture with APV from 3 (a), 6(b), 9(c), and 11 DIV (d) to 16 DIV, and from 0 to 6 DIV (e) and 6 to 11 DIV (f).

EXPERIMENTAL PROCEDURES

Organotypic slice culture

Details are given elsewhere (Takuma et al., 2002). In brief, coronal slices (400 μm) of sensorimotor cortex and axial slices (400 μm) of the cervical cord from P0 Wister rats were sectioned, and the forelimb areas of the cortex excised from each section with a razor blade. The cortical and spinal cord slices were placed on collagen-coated membranes (Transwell-Col, 3.0 μm pore; Costar, Cambridge, MA, USA); then serum-free, hormone-supplemented medium (Dulbecco's modified essential medium/Ham's F-12 with 100 μg/ml transferrin, 20 nM selenium, 20 nM hydrocortisone, 20 nM progesterone, and 5 μg/ml insulin) was added. Fetal bovine serum (5%) was added for the first 3 days. Cultures were maintained at 37 °C in humidified 95% air and 5% CO_2. The level of the medium was adjusted to slightly below the surface of the explants (Yamamoto et al., 1989; Yamamoto and Toyama, 1995).

All animal experiments were performed in accordance with the Ethical Committee Guidelines for Animal Experimentation, Teikyo University School of Medicine and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996. All efforts were made to minimize the number of animals used and their suffering.

Electrophysiological study

The NMDA receptor antagonist, 50 μM APV (Sigma, St. Louis, MO, USA) was added to the medium at 0, 3, 6, 7, 8, 9, 10, 11, 14 DIV until the end of culture, or from 0–6 and 6–11 DIV (Fig. 3a).

Slices were placed in a recording chamber and perfused with standard medium containing (in mM) NaCl 119, KCl 2.5, NaH_2PO_4 1, $NaHCO_3$ 26, $MgSO_4$ 1.29, $CaCl_2$ 2.42, and equilibrated with a 95% O_2/5% CO_2 gas mixture (Sakurai, 1987). They were transferred from the drug-added medium to the recording chamber and washed for more than 30 min with perfusate before recordings. A stimulating bipolar electrode was placed in the deep layer of each cortical slice, and current pulses (amplitude 500 μA, width 100 μs) were applied to stimulate axons projecting to the spinal cord. fEPSPs were recorded with a glass micropipette filled with 2 M NaCl (2–3 MΩ; Ito et al., 1982) along a 100 μm-interval lattice within the spinal gray matter ipsilateral to the stimulating electrode. fEPSPs were recorded at 16 DIV.

Morphological study

Biocytin (Sigma) was placed on the deep layers of cortical slices after 14 DIV to label axons anterogradely and examine cortical axon extension into the spinal cord, after which the slices were incubated for 48 h then fixed with 4% paraformaldehyde and 0.5%