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COINCIDENT STIMULATION OF CONVERGENT CORTICAL INPUTS ENHANCES IMMEDIATE EARLY GENE INDUCTION IN THE STRIATUM

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Abstract—The effect of coincident stimulation of convergent corticostriatal inputs was analyzed by the induction of immediate early genes in striatal neurons. Cortical motor areas were stimulated through implanted electrodes in awake, behaving rats, and the induction of the mRNAs encoding the immediate early genes (IEGs) *c-fos* and *arc* was analyzed in the striatum with *in situ* hybridization histochemistry. In the first experiment, unilateral stimulation of the medial agranular cortex, orofacial region of the lateral agranular cortex or the forelimb region of the lateral agranular cortex resulted in IEG induction in the striatum, which was restricted to the topographically related area receiving input from the stimulated cortical area. In a second experiment, stimulation parameters were altered, including frequency, number of pulses/train, and number of trains/s. These parameters did not have a significant effect on IEG induction. Notably, in some cases, in which there was IEG induction not only in the stimulated cortical region, but also in the homologous area in the contralateral hemisphere, very robust IEG induction was observed in the striatum. In a third experiment, the orofacial regions of the lateral agranular cortex of both hemispheres were stimulated coincidently. All of these animals showed robust striatal IEG induction. This IEG induction was attenuated by pretreatment with an NMDA antagonist MK-801. In a fourth experiment, we tested whether the coincidence of bilateral cortical stimulation contributed to the efficacy of striatal IEG induction. Either alternating stimulation or coincident stimulation of non-homologous cortical regions produced significantly lower striatal IEG induction than obtained with coincident stimulation of homologous cortical areas. Enhanced striatal IEG induction occurred in indirect striatal neurons, labeled with enkephalin, but was also present in a large number of enkephalin-negative neurons, most of which are likely direct pathway neurons. These results suggest that regional and temporal convergence of cortical inputs enhances striatal IEG induction. Published by Elsevier Ltd on behalf of IBRO.

Key words: basal ganglia, gene regulation, glutamate, corticostriatal, neuronal plasticity.

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Abbreviations: AGl-fl, forelimb area of the lateral agranular cortex; AGl-o, orofacial area of the lateral agranular cortex; AGm, medial agranular cortex; IEG, immediate early gene; ISHH, *in situ* hybridization histochemical; RD, relative density.

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Involvement of the basal ganglia in motor learning was suggested by both clinical (Knopman and Nissen, 1991; Knowlton et al., 1996) and animal (Miyachi et al., 1997; Matsumoto et al., 1999) studies, which have shown that dysfunction of the basal ganglia results in disorders of procedural or skill learning. Learning-related activities were also demonstrated by imaging studies in human subjects (Rauch et al., 1997; Jueptner and Weiller, 1998) and by electrophysiology in non-human primates (Aosaki et al., 1994; Miyachi et al., 2002).

Learning dependent changes in synaptic plasticity involve changes in gene regulation, which are mediated, in part, by the induction of immediate early genes (IEGs) (Morgan and Curran, 1989; Sheng and Greenberg, 1990). Recently, several reports have shown that IEGs including *c-fos* and *arc* are induced in the brain by learning in behaving animals (Grimm and Tischmeyer, 1997; Guzowski et al., 2001; Kelly and Deadwyler, 2002) and by synaptic plasticity in slice preparations (Abraham et al., 1993). Ying et al. (2002) showed that upregulation of the IEG *arc* is necessary for hippocampal long-term potentiation triggered by the brain-derived neurotrophic factor.

The commonly studied forms of synaptic plasticity, LTP and LTD, have been elicited within the striatum in response to corticostriatal stimulation (Calabresi et al., 1992, 1994; Kerr and Wickens, 2001; Reynolds and Wickens, 2000). Moreover, stimulation of corticostriatal inputs results in the induction of IEGs within the striatum (Fu and Beckstead, 1992; Wan et al., 1992; Liste et al., 1995; Parthasarathy and Graybiel, 1997; Sgambato et al., 1997), which is mediated through activation of the ERK1-2 MAP-kinase signaling pathway (Sgambato et al., 1998; Gerfen et al., 2002). Corticostriatal axons from individual cortical neurons provide extremely sparse input to individual striatal neurons (Kincaid et al., 1998; Zheng and Wilson, 2002). Thus, activity in striatal medium spiny neurons depends on the convergent inputs of many corticostriatal neurons. Corticostriatal inputs from homologous cortical areas or from somatotopically related areas of different cortical areas converge within the striatum (Wilson, 1986; Parthasarathy et al., 1992; Flaherty and Graybiel, 1993; Inase et al., 1996). Prior studies of corticostriatal stimulation resulting in IEG induction have utilized stimulation of single cortical sites. One question is whether stimulation of convergent corticostriatal inputs might enhance striatal IEG induction. Another important question is which subclass of striatal projection neurons expresses IEGs following cortical stimulation. Striatal projection neurons, which account for more than 90% of the striatal neuronal population, are divided to two subclasses on the basis of differ-

ences in their axonal projections (Kawaguchi et al., 1990) and expression of neuropeptides and dopamine receptor subtypes (Gerfen et al., 1990). In some studies, it has been reported that corticostriatal activation results in IEG induction mainly in the indirect pathway neurons (Berretta et al., 1997; Parthasarathy and Graybiel, 1997), whereas other studies did not find a significant difference between direct and indirect pathway neurons (Sgambato et al., 1997).

To answer these questions, we stimulated the rat motor cortex with implanted electrode(s) while the animals were awake, and examined striatal IEG induction. We show that electric stimulation of the cortical motor areas induced IEGs *c-fos* and *arc* in the corresponding areas in the striatum. We also report that striatal IEG induction 1) reflects convergence of two cortical inputs, rather than intensity of input from one cortical area, 2) depends on NMDA receptor activation, and 3) occurs mainly in indirect pathway neurons, but also in other striatal neurons, which are likely direct pathway neurons.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (Taconic, Germantown, NY, USA), weighing 250–350 g, were used. Rats were housed in groups of two or three in a temperature-controlled room on a 12-h light/dark schedule. Rats had free access to food and water. Experiments conformed to National Institutes of Health guidelines for the care and use of laboratory animals, which minimize both the number of animals used and their pain and distress in the conduct of the studies.

Cortical electrical stimulation and drug application

All animals were stimulated through monopolar electrode(s) surgically implanted in the motor cortex. The coordinates of electrodes were as follows: 3 mm anterior, 3.5 mm lateral to bregma (orofacial area of the lateral agranular cortex, AGl-o), 0.5 mm anterior, 1 mm lateral (forelimb area of the lateral agranular cortex, AGl-fl), or 3 mm anterior, 1 mm lateral to bregma (medial agranular cortex, AGm).

Three to 7 days after surgery, rats were placed in individual experimental chambers for stimulation. The rats were allowed to stay with experimental setup for at least 2 h for habituation, then stimulated with pulse trains for 30 min. The animals were killed with CO_2 and decapitation immediately after the stimulation offset. Brains were removed and rapidly frozen on dry ice.

The animals were stimulated with biphasic pulse trains (0.3 ms pulse duration) generated by a stimulator (FHC, Inc, ME, USA). The typical parameters were 100 Hz, 160 ms trains, and one train per second, but several different sets of parameters were also used (see Results). Stimulation was continued for 30 min. The current intensity was 100 μA in all cases. This intensity was chosen so as to elicit reliable somatic movement, but not a generalized seizure, in most cases. Cases in which test stimulation (with single pulse trains at the same frequency and train duration as in the experiments) induced no visible movements were excluded from the analysis.

For blockade of NMDA receptors, a selective antagonist MK-801 (5 mg/kg) (Sigma, St. Louis, MO, USA) was injected i.p. 10 min before the stimulation onset.

Histological procedures

Immediately after the animals were killed, the brains were removed and frozen on dry ice. Brains were cut in 12-μm-sections on a cryostat. Sections were thaw-mounted onto gelatin-chrome alum-subbed slides and stored at −20 °C. All slides were post-fixed in 4% formaldehyde in 0.9% NaCl, acetylated in fresh 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl, dehydrated in an ascending series of ethanol, delipidated in chloroform, and then rehydrated in a descending series of ethanol. Slides were air dried and then stored at −20 °C.

For detection of *arc* and *c-fos* mRNAs, sections were hybridized with ^{35}S-labeled oligonucleotide probes overnight using a method described previously (Gerfen et al., 1995). Briefly, 48 base oligonucleotide probes antisense to a region in the 5′ UTR of *c-fos* (Curran et al., 1987) and *arc* (Lyford et al., 1995) were end labeled with ^{35}S- dATP. Probes were diluted to 2×10^6 cpm/100 μl of hybridization buffer containing: 0.6 M NaCl, 80 mM Tris (pH 7.5), 4 mM EDTA, 0.2% sodium dodecyl sulfate, 0.1% (wt/vol) sodium pyrophosphate, 10% dextran sulfate, 0.02% heparin sulfate, 50% formamide, and 100 mM dithiothreitol. 100 μl of probe in hybridization buffer was applied to each slide on which there were three to four brain sections, coverslipped and incubated overnight at 37 °C. Slides were then washed four times in 1× SSC with 50% formamide at 40 °C for a total of 1 h, rinsed in 1× SSC for two 30 min periods, rinsed in water and air dried. Labeled slides were apposed to X-ray film for 3 days to 3 weeks.

To determine the subtypes of the striatal projection neurons in which IEGs were induced following cortical stimulation, a double fluorescent *in situ* hybridization histochemical (ISHH) procedure was used (Guzowski et al., 2001). Using this procedure, an antisense enkephalin ribonucleotide probe (450 bases) was prepared by labeling with UTP-FITC and an antisense *arc* ribonucleotide probe (1100 bases, Lyford et al., 1995) was prepared by labeling with UTP-digoxigenin. These probes were mixed together with hybridization buffer (50% formamide, 600 mM NaCl, 80 mM Tris–HCl, pH 7.5, 4 mM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 2% sodium polyacrylate, 100 mM dithiothreitol; 1 μg tRNA; 1 μg total RNA; 0.4 μg salmon sperm DNA), applied to the glass-mounted sections, and incubated at 55 °C overnight. After treatment with RNAase A (20 mg/ml) for 30 min, slides were then washed for 4×20 min at 65 °C in 0.2× SSC, rinsed in Tris (0.5 M, pH 7.5) saline (0.9%) at room temperature for 5 min. *Arc* probe was detected with an antidigoxigenin–horseradish peroxidase conjugate (Roche Molecular Biochemicals Indianapolis, IN, USA) and the TSA-direct Cyanine-3 kit (NEN Life Sciences, Boston, MA, USA). (For detail, see Guzowski et al., 2001.)

mRNA expression analysis

To compare the relative density (RD) of the IEG induction in different animals, sections to be compared were hybridized with the same batch of mRNA probe and put on X-ray film for the same period of time. Images were captured from films using a CCD camera, together with National Institutes of Health Image software (W. Rasband, National Institute of Mental Health). Average gray values from an area of a fixed size (1 mm^2) were measured at the region of interest (lateral-most part of the striatum at the AP level of the bregma) and control striatal region (dorsomedial edge of the striatum in the same section). The RD was calculated as (gray value of the region of interest)−(gray value of the control region). RD for different stimulation parameters were compared with ANOVA and Scheffe post-hoc test.

RESULTS

Cortical stimulation in awake rats induced striatal *arc* in a topographical manner

Three cortical motor areas were electrically stimulated (100 Hz, 160 ms, one train/s, 30 min) in awake rats; AGl-o, AGl-fl, and AGm. Characteristic movements were induced