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Expression of c-*fos* Protein in Brain: Metabolic Mapping at the Cellular Level

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The proto-oncogene c-*fos* is expressed in neurons in response to direct stimulation by growth factors and neurotransmitters. In order to determine whether the c-*fos* protein (Fos) and Fos-related proteins can be induced in response to polysynaptic activation, rat hindlimb motor/sensory cortex was stimulated electrically and Fos expression examined immunohistochemically. Three hours after the onset of stimulation, focal nuclear Fos staining was seen in motor and sensory thalamus, pontine nuclei, globus pallidus, and cerebellum. Moreover, 24-hour water deprivation resulted in Fos expression in paraventricular and supraoptic nuclei. Fos immunohistochemistry therefore provides a cellular method to label polysynaptically activated neurons and thereby map functional pathways.

THE C-*fos* GENE, THE NORMAL CELLULAR counterpart of the viral oncogene v-*fos* (*1*), is rapidly and transiently expressed in many tissues in response to growth factor stimulation (*2*). It encodes a nuclear phosphoprotein (Fos) that exhibits both nonspecific (*3–5*) and sequence-specific (*6*) DNA binding properties. A set of Fos-related antigens has been identified that also appear to be nuclear DNA binding proteins (*4, 7, 8*). Although the function of c-*fos* is not known, it has been suggested that it acts as a "third messenger" molecule in signal transduction systems, where it would couple short-term intracellular signals elicited by a variety of extracellular stimuli to long-term responses by altering gene expression (*9*).

Fos can be demonstrated immunohistochemically to be in the nuclei of neurons in normal and pathological circumstances (*10–12*). In vitro, c-*fos* RNA and Fos rapidly increase in pheochromocytoma (PC12) cells after depolarization or stimulation with cholinergic agonists (*13*). Fos is normally present in rodent brain, particularly cerebral cortex and hippocampus, and its expression increases transiently after generalized seizures (*11, 12*). Moreover, cutaneous stimulation induces Fos expression in neurons of the spinal cord dorsal horn (*10*).

This literature suggests that Fos is transiently expressed in neurons after synaptic stimulation. Since the basal expression of Fos is relatively low in most central nervous system (CNS) regions, Fos expression might provide an anatomic technique for metabolic mapping similar in some respects to 2-deoxyglucose autoradiography (*14*). Although lacking the quantitative potential of the 2-deoxyglucose method, Fos expression offers cellular resolution, since immunohistochemical Fos staining is localized to the cell nucleus.

Bipolar stimulating electrodes were implanted into the hindlimb motor/sensory cortex of adult rats that were under general anesthesia (*15*). Twenty-four hours later, electrical stimulation was carried out in four awake, restrained animals for 15 min or for 1 hour in one animal (*16*). Two sham-operated animals were restrained, but received no electrical stimulation. Two unoperated, unanesthetized animals were also examined. Three hours after the beginning of stimulation, rats were anesthetized with pentobarbital, 50 mg/kg intraperitoneally, and were perfused through the aorta with saline and then with 4% paraformaldehyde in 0.1*M* sodium phosphate buffer, *p*H 7.4 (PB). Brains were removed and immersed in the same fixative for 4 hours at 4°C and then washed in PB. Fifty-micrometer coronal sections were cut on a Vibratome and were stained immunohistochemically for Fos by the avidin-biotin-peroxidase (ABC) method (*17*). The primary antiserum is an affinity-purified antiserum raised to a synthetic peptide, the M peptide (*5, 7*), corresponding to residues 127 to 152, which are common to both c-*fos* and v-*fos*. This antiserum has been characterized (*5, 7*) and cross-reacts with several Fos-related antigens (*4, 5, 7, 8*).

In control brains into which electrodes were inserted but no current passed, there was staining of cell nuclei in a reproducible and symmetric pattern in cerebral cortex (predominantly layers 1 to 4), hippocampal formation, striatum, piriform cortex, amygdala, and basal forebrain. The cerebellum, brainstem, thalamus, and globus pallidus of control brains were mainly devoid of staining, except for scattered cells. This is in agreement with previous observations (*12*).

The brains of stimulated rats exhibited two major differences from control brains. First, all stimulated subjects had increased staining in the motor/sensory cortex compared to controls, though only in three of five was the staining asymmetrically increased on the stimulated side (Fig. 1A) compared to the contralateral side (Fig. 1B). Second, in all stimulated animals there were focal areas of asymmetric increased Fos staining corresponding to brain regions known to be targets of motor/sensory cortex output. These regions include ipsilateral thalamus in the ventrolateral, ventrobasal, centrolateral, mediodorsal, posterior, parafascicular, ventromedial, and reticular nuclei (Fig. 1, C and D). In one animal a few cells in the ipsilateral globus pallidus expressed Fos nuclear staining (*18*). Focal staining of cells in ipsilateral pontine nuclei was also seen (Fig. 2C).

Of particular note are areas of nuclear staining in the copula pyramidus of the cerebellum, most marked contralateral to the stimulation, but with less dense staining in the corresponding ipsilateral area as well (Fig. 2A). These regions appear to correspond to cerebellar microzones. These are zones 0.5 to 1 mm long in cerebellar cortex, which can be defined electrophysiologically, anatomically by afferent inputs, or anatomically by neurotransmitter distribution (*19*). They may represent regions in which different functional modalities are integrated within the cerebellum (*20*). The vast majority of stained nuclei in the cerebellum were

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small nuclei in the granule cell layer; Purkinje cells displayed cytoplasmic staining inside (*21*) as well as outside (Fig. 2B) the activated granule cell microzones.

All of these areas of increased Fos staining correspond to regions known from prior 2-deoxyglucose experiments to be metabolically activated by a stimulation protocol identical to the present one (*20, 22*). The increased Fos expression in the cerebellar cortex is of particular note, because these cells must be at least two synapses from the directly stimulated cortical cells (*23*) and because of the correlation between Fos immunostaining and 2-deoxyglucose autoradiographs (*20, 22*).

There are also regions that have been shown to have increased glucose metabolism in response to electrical stimulation of motor/sensory cortex, but which have no detectable change in Fos expression. Possibly because basal Fos staining of striatum is high, focal areas of increased Fos staining were not noted in the caudate/putamen of stimulated animals. In one animal, there was a generalized asymmetry of striatal Fos staining, with darker staining ipsilateral to the stimulation. Also, the stimulation site itself shows only a moderate increase in Fos staining compared to surrounding cortex, whereas the site displays dramatic increased glucose utilization.

In addition to the focal areas of Fos staining, there is a generalized increase in the intensity of immunostaining of neuronal nuclei in the granule cells of the dentate gyrus and in hippocampal pyramidal cells (Fig. 3, A and B). Moreover, although in some animals cortical neurons ipsilateral to the stimulation stained more darkly than those contralateral, both sides were more darkly stained than unstimulated cortex in all experimental subjects. These hippocampal and cortical patterns are similar to those seen after Metrazole (pentylenetetrazole) induced or kindled generalized seizures (*11, 12*). The rats that receive focal electrical stimulation of motor/sensory cortex have intermittent bilateral motor activity, probably representing secondary generalization of their focal hindlimb motor activity. This may account for the similarity of staining pattern to that seen in other types of generalized seizures. Thus, similar patterns of Fos expression are produced by dissimilar methods of inducing seizures.

Both sham-operated and stimulated animals exhibited variable Fos staining of the paraventricular and supraoptic nuclei of the hypothalamus, the cell bodies of origin of the hypothalamo-neurohypophysial tract. We hypothesized that this increase was due to the failure of the rats to drink normally after anesthesia. We therefore compared four unoperated rats deprived of water for 24 hours with three control rats given free access to water. Fos staining markedly increased in the paraventricular (magnocellu-

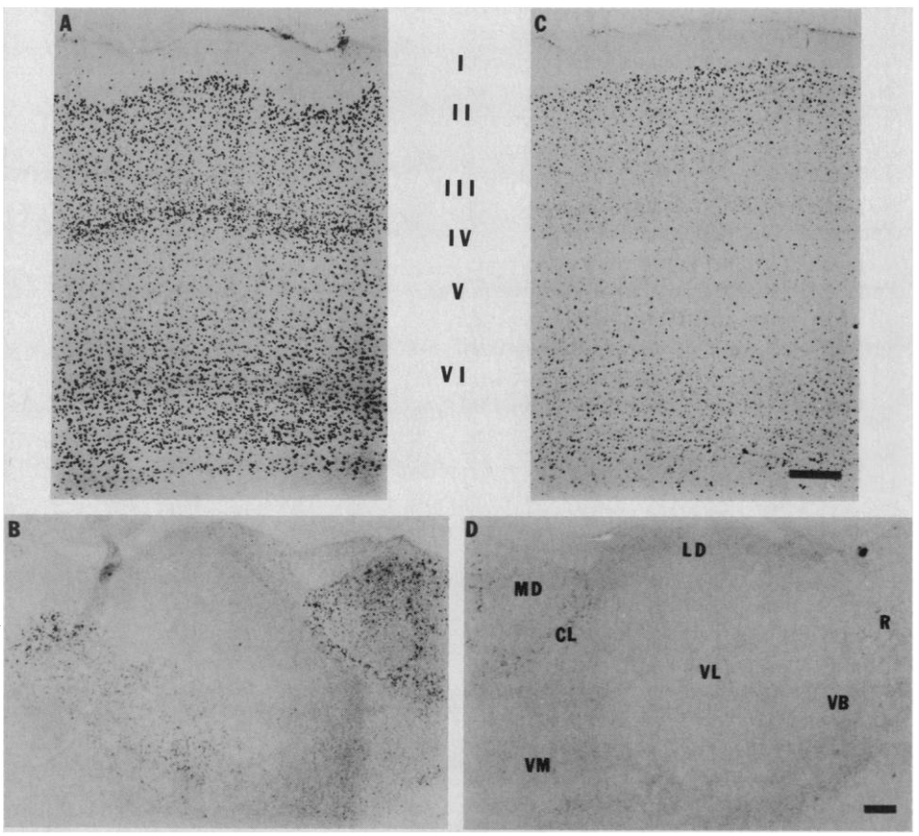

Fig. 1. Coronal sections of rat cerebral cortex (**A** and **C**) and thalamus (**B** and **D**) immunostained for Fos (*17*). (A) and (B) are ipsilateral to the stimulation site in left hindlimb motor/sensory cortex, and (C) and (D) are contralateral. The Fos staining is localized to the nuclei of neurons. The cortical layers are indicated in (A) and (C) by roman numerals. Thalamic nuclei are labeled as follows: CL, centrolateral; LD, lateral dorsal; MD, mediodorsal; R, reticular; VB, ventrobasal; VL, ventrolateral; and VM, ventromedial. Scale bars, 200 μm.

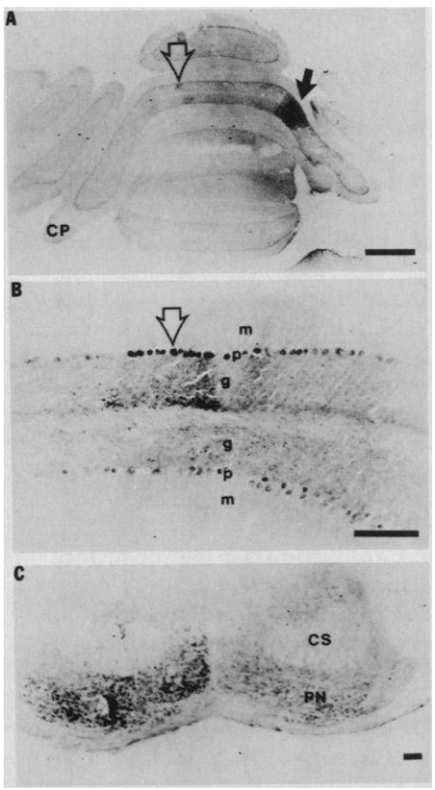

Fig. 2. Fos-stained coronal sections of cerebellum (**A** and **B**) and basis pontis (**C**) from a rat stimulated in left hindlimb motor/sensory cortex. In (A) the filled arrow marks a microzone that shows increased Fos staining contralateral to the stimulation site. The open arrow shows a region of less dense staining ipsilateral to the stimulation. (B) A higher power photomicrograph of the region marked by the open arrow in (A). The staining in the granule cell layer appears nuclear, although it is not possible to be sure of this in the light microscope. Purkinje cell staining (B) is primarily cytoplasmic and may therefore represent a c-*fos*–related protein, rather than Fos itself. (C) Dense nuclear Fos staining of pontine neurons ipsilateral to the stimulation (on the left of the figure) is seen. Abbreviations: CP, copula pyramidus; CS, corticospinal tract; g, granule cell layer; m, molecular layer; p, Purkinje cell layer; and PN, pontine nuclei. These sections were treated with 1% OsO_4 for 3 min to intensify staining. Both the nuclear staining in the pons and granule cell layer of the cerebellum and the cytoplasmic staining of Purkinje cells are completely blocked by inclusion of M peptide, 2 μg/ml, in the incubation with primary antiserum. Scale bars, in (A), 1 mm; in (B) and (C), 200 μm.

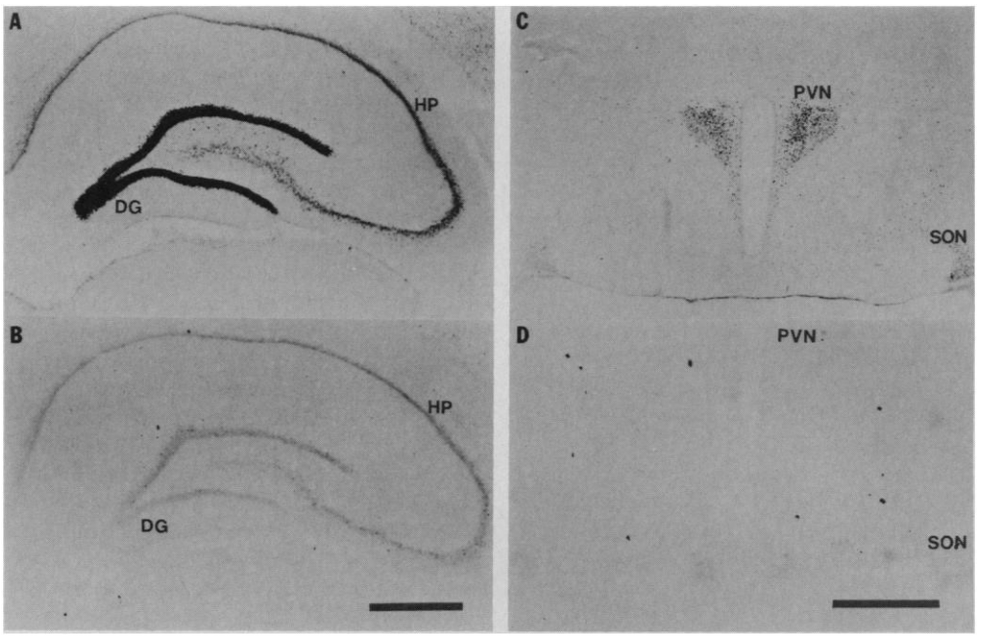

Fig. 3. Coronal sections through the hippocampus of stimulated (**A**) and unstimulated (**B**) rats. Note the extremely dense Fos immunostaining of dentate granule cells and of many hippocampal pyramidal cells in the stimulated animal. Coronal sections through the anterior hypothalamus of water-deprived (**C**) and control (**D**) rats demonstrating dehydration-induced Fos expression in the paraventricular and supraoptic nuclei. Abbreviations: DG, dentate granule cells; HP, hippocampal pyramidal cells; PVN, paraventricular nucleus; and SON, supraoptic nucleus. Scale bars, 1 mm.

lar and parvocellular divisions) and supraoptic nuclei of the water-deprived rats (Fig. 3C) as compared to control rats (Fig. 3D). Therefore, water deprivation increases Fos staining in those hypothalamic neurons known to be activated by dehydration (*24*). This is in contrast to the 2-deoxyglucose technique, which does not demonstrate marked increased glucose utilization in the hypothalamic magnocellular nuclei in spite of known increases in the firing rate of these cells (*25, 26*).

Our observations, along with those of Hunt *et al.* (*10*), demonstrate that Fos immunohistochemistry labels at least some subset of physiologically activated neurons. Hunt *et al.* (*10*) found Fos staining in dorsal horn neurons of the spinal cord in response to various modalities of cutaneous stimulation. The neurons expressing Fos immunoreactivity are the presumed second-order relay neurons of the primary afferents stimulated. However, Fos staining was not detected in third-order neurons of brainstem. In contrast, we find stimulation of Fos staining across at least two synapses in response to stimulation of cerebral cortex, most consistently in the cerebellum. The difference between the two studies may relate to the use of antibodies directed at different peptides derived from Fos, strength or duration of stimulation, the time after stimulation at which brains were examined, or biologic differences between the systems examined.

The differences between the patterns of Fos immunostaining observed in the experiments reported here as compared to corresponding 2-deoxyglucose autoradiographs can be explained by several factors. Fos is a nuclear stain, so that regional Fos expression only marks activated cell bodies. Autoradiographs with 2-deoxyglucose, in contrast, provide a measure of glucose utilization throughout the cell and may be more sensitive to alterations in functional activity in the neuropil than in cell bodies (*26, 27*). This explains at least in part the difference between the two techniques in the examination of the response to dehydration and also may explain some of the differences between the two techniques in regional patterns of response to electrical stimulation of cerebral cortex. In addition, we examined animals at only one time interval after electrical stimulation; it is possible that the patterns of Fos expression vary with time.

The 2-deoxyglucose method measures regional metabolic activity and maps functional activity primarily in neuropil. In general, however, the method cannot distinguish between altered glucose metabolism of neurons intrinsic to a brain region from that of terminals of afferent fibers. Since Fos immunohistochemistry stains the nuclei of activated cells, it allows the identification of specific neurons. These cells may be characterized by location, neurotransmitter content, cytologic appearance, or any other morphologic feature whose analysis is compatible with immunohistochemistry.

The primary uncertainty of the Fos method of metabolic mapping is lack of knowledge concerning the range of stimuli that will produce alterations of Fos synthesis in the CNS. There may well be situations, as illustrated by the work of Hunt *et al.* (*10*), in which Fos immunostaining fails to adequately map functional pathways. The same limitation applies to 2-deoxyglucose and will likely apply to subsequent techniques as well. Further work will be necessary to determine the specific factors responsible for induction of Fos expression by synaptic activity and the physiologic function of Fos in the CNS. Nevertheless, it provides a cellular method for labeling neurons activated by a variety of stimuli, potentially having widespread application in neurobiological research.

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15. Adult male Long-Evans and Sprague-Dawley rats receive ketamine, 80 mg/kg intraperitoneally, and xylazine, 8 mg/kg intraperitoneally. Each rat is placed in a stereotaxic frame with blunt ear bars, and the scalp is incised and a burr hole drilled in the skull. A coaxial electrode with inner contact diameter of 200 μm and an outer contact diameter of 500 μm and 500 μm between contacts is lowered under stereotaxic control into hindlimb motor cortex. The electrode is cemented into place with dental acrylic and the scalp incision is closed. The scalp is infiltrated with Xylocaine, and the animal is returned to its cage with free access to food and water.
16. Rats are restrained and stimulated with a Grass Constant Voltage Stimulator with 5 trains per second of 20-ms trains of 300 Hz, 0.5-ms biphasic pulses. The current is adjusted to produce hindlimb movements without generalized convulsions, although most animals display intermittent bilateral motor activity. We noted no consistent differences in Fos staining pattern between 15 min and 1 hour of stimulation. After stimulation the animals are again returned to their cages.
17. Sections were incubated for 30 min at room temperature in 0.1*M* PB, *p*H 7.4, 0.2% Triton X-100, 0.1% bovine serum albumin, and 2% normal goat serum (PB-G). Sections were then incubated for 36 to 48 hours at 4°C with gentle agitation in primary antiserum diluted 1/100 in PB-G. After washing three times in PB for a total of 30 min, sections were processed with a Vectastain ABC Kit (Vector Labs) with secondary antiserum and ABC reagent dissolved in PB-G. Incubation times were 2 to 3 hours at room temperature. Diaminobenzidine is used as the chromagen. Adsorption controls with synthetic M peptide, 2 μg/ml, included in the incubation with primary antiserum showed no nuclear staining.
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Familial Imprinting Determines H-2 Selective Mating Preferences

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Inbred male mice typically prefer to mate with females of a different, non-self H-2 haplotype. To determine whether this natural preference is irrevocable or results from familial imprinting, a test system was used which relied on previous observations that B6 males (H-2^b) mate preferentially with congenic B6–H-2^k rather than B6 females, and B6–H-2^k males with B6 females. This preference was reversed in B6 males fostered by B6–H-2^k parents and in B6–H-2^k males fostered by B6 parents, preference in these cases favoring the same H-2 type. Thus, H-2 selective mating preference is acquired by imprinting on familial H-2 types.

INBRED MALE MICE SHOW A TENDENCY to mate with females of an H-2 type different from their own (*1*); for example, when presented with equivalent C57BL/6 (B6; H-2^b) and B6–H-2^k females in estrus, a B6 male more often selects the B6–H-2^k female and a B6–H-2^k male more often selects the B6 female (*2*), as illustrated in Table 1, group 1.

To determine whether this natural preference for the non-self H-2 haplotype is acquired during early life, we studied the mating preferences of B6 males reared by B6–H-2^k foster parents and of B6–H-2^k males reared by B6 foster parents. Although the genetic relationship is unnatural, since parents of the same homozygous H-2 genotype cannot give birth to homozygous progeny of a different H-2 type, this experimental design seemed most likely to reveal any influence that imprinting of parental H-2 types may have with respect to subsequent choice of a mate. The idea that imprinting of parental odors affects the subsequent behavior of mice is not new since it has been invoked, though not in relation to particular genes, to explain observed influences of artificial scenting of sires (*3*), removal of sires (*3*), or foster-nursing (*4*) on subsequent social or mating proclivities. Our present finding that exposure history critically determines H-2–based male mating preference implies that avoidance of mating with closely related individuals (kin) is determined neither by a direct genetic mechanism (recognition alleles) nor by use of self H-2 as a referent (*5*).

Within 16 hours of birth, entire litters were removed from their natural parents and transferred to foster parents whose own litters, born at approximately the same time, were simultaneously removed. At 21 days of age, the fostered mice were weaned and the males maintained in stock cages containing only males of the same genotype and fostering history until sexual maturity (3.5 months of age, minimum), when tests of mating preference began.

The method of testing mating preference was as described (*2*) except that the males in the present studies had been reared by foster parents. A fostered male, B6 or B6–H-2^k, vasectomized at 2 months of age to avert unwanted pregnancies and housed alone in an individually ventilated cage, was presented with two females in estrus that had been selected from congenic mouse panels, B6 and B6–H-2^k, each of 60 to 80 individually numbered age-matched females. In each test the two estrous females were selected from the panels with due attention to equality of their previous sexual experience in repeated testing of the males. The two females were placed in the male's cage and watched until successful copulation was verified by presence of a vaginal plug, or until 2 hours had elapsed. Each test was scored as valid only if the second female was shown to be in estrus and receptive to copulation, as verified by vaginal plug, with a male from a separate panel of males maintained for this purpose. The data given include only fostered males that achieved two valid tests, as defined above, within no more than three trials. The minimum interval between testing of males was 10 days.

To provide a control to verify the segregation of scent individuality with H-2, thus excluding hypothetical influences of genetic drift affecting loci other than H-2 and of any nongenetic distinguishing characteristics that might have arisen since the B6–H-2^k strain was originally derived, the B6 and B6–H-2^k strains were first rederived from a

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