Transneuronal Transfer of Herpes Virus from Peripheral Nerves to Cortex and Brainstem

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The transneuronal transfer of neurotropic viruses may represent an effective tool for tracing chains of connected neurons because replication of virus in the recipient neurons after transfer amplifies the "tracer signal." Herpes simplex virus type 1 was transferred transneuronally from forelimb and hindlimb nerves of rats to the cortical and brainstem neurons that project to the spinal enlargements to which the nerves receiving injections are connected. This transneuronal transfer of herpes simplex virus type 1 from peripheral nerves has the potential to be used to identify neurons in the brain that are related transsynaptically to different nerves and muscles.

THE TRANSNEURONAL TRANSFER OF tracer substances between connected neurons would represent a simple method for examining complex neuronal organizations, such as the chains of neurons linking the brain with the periphery. For a substance to be effective as a transneuronal tracer, transfer should occur preferentially at synapses, and enough tracer should reach the recipient neurons to allow detection. Some toxins (*1*), lectins (*2*), and neurotropic viruses (*3–5*) share these characteristics. Neurotropic viruses are replicated in neurons after transneuronal transfer, thus enhancing the signal (*4, 5*). In the present study, a striking demonstration of transneuronal transfer was observed. Injection of the neurotropic herpes simplex virus 1 (HSV-1) into forelimb and hindlimb nerves of rats produced intense transneuronal labeling of cortical and brainstem neurons (Fig. 1) at sites that project to the spinal segments to which the nerves receiving injections are connected. The virus was transferred transneuronally from peripheral nerves to the contralateral sensorimotor cortet and specifically from the forelimb and hindlimb nerves to the forelimb and hindlimb cortical areas. These findings support the notion of Kristensson and co-workers (*3*) that the propagation of HSV-1 through the brain involves transneuronal transfer and emphasize that this propagation may occur over long distances.

HSV-1 (strain SC16) (2 to 4 μl; titer, 5×10^9 plaque-forming units per milliliter) was injected unilaterally into the ulnar and median nerves of eight rats (age 6 to 7 weeks) (*6*). In two (control) rats the injections were preceded by transection of the nerves proximal to the injection sites. In four additional rats, HSV-1 (2 to 3 μl) was injected unilaterally into the tibial nerve. After 3.5 to 4 days all animals were deeply anesthetized and perfused with 10% Formalin (*4*). Brains and spinal cords were cut transversely in frozen sections (60 μm). The virus was demonstrated immunohistochemically by the peroxidase-antiperoxidase method (*4*).

Many strongly labeled neurons were observed in the brains of the experimental animals, whereas no labeled neurons were found in the controls. Therefore, these neurons must have received virus from the injected nerves. Labeled neurons were concentrated in those cortical and brainstem areas that project to the spinal cord (*7–18*). After ulnar and median injections, strongly labeled neurons were present in layer V of the forelimb area of the contralateral sensorimotor cortex (*8, 19, 20*) (Figs. 1A and 2). These neurons were largely pyramidal in shape, with prominent apical dendrites (Fig. 1A). A few neurons were found in the dorsal portion of the contralateral red nucleus (Fig. 2B). These cortical and red nucleus areas project to the contralateral cervical gray matter (*8, 10*) and steer contralateral forelimb movements (*19–21*).

Many labeled neurons were also present in the hypothalamic areas that project to the spinal cord (for example, paraventricular nucleus and lateral hypothalamus) (*11, 17*) (Figs. 1B and 2A) and at multiple brainstem sites, including the raphe nuclei in the lower brainstem and the adjoining ventral and ventrolateral reticular formation (Fig. 2, D to F), as well as the nuclei locus coeruleus, subcoeruleus, and Kölliker-Fuse at pontine levels (Fig. 1C and Fig. 2, C and D). Other areas containing labeled neurons included midline nucleus of Edinger-Westphal (Fig. 2B), periaqueductal gray, ventrolateral pontine tegmentum (Figs. 1C and 2D), the area lateral to the superior olives, and the base of the cuneate nucleus (Fig. 2G). All these cell groups project to the spinal cord (*10–18*). At survival times longer than 4 days the infection became more extensive; labeled neurons became more numerous and were surrounded by an increasing number of glial cells.

In the spinal cord, virus antigen was concentrated in low cervical and upper thoracic segments on the side of the injection (Fig. 3). A dense accumulation was found in the dorsal horn, except its lateral part, and in the dorsal funiculus, in which it could be traced to upper cervical levels. Antigen was also present in the intermediate zone (laminae V to VII) (mostly absent in lamina VIII) and in lateral motoneuronal cell groups of the ventral horn, including in several cases the area of the autonomic lateral horn at upper thoracid levels. Some antigen was present in the ventral funiculus traversed by ventral root fibers.

Virus injections into tibial nerve produced a different distribution of labeling. In the spinal cord, antigen was present in the lumbar and sacral segments, where it was distributed approximately as in cervical-thoracic segments of ulnar and median cases. In the brain, the positive neurons in the sensorimotor cortex (Fig. 2, asterisks) were cen-

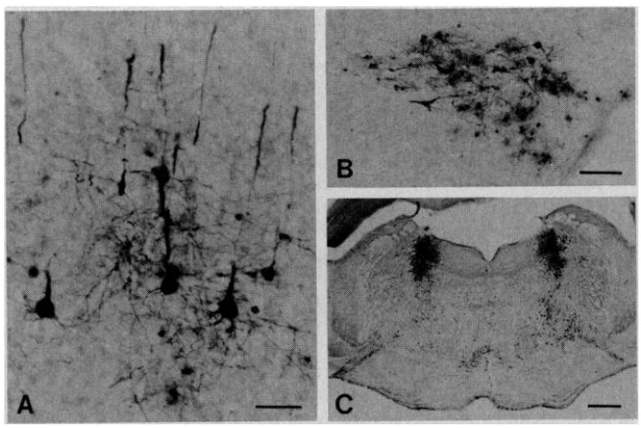

Fig. 1. Labeled neurons (immunohistochemical staining) (*4*) after unilateral HSV-1 injection into ulnar and median nerves. (**A**) Pyramidal neurons in layer V of contralateral sensorimotor cortex. Basal and apical dendrites are shown (scale bar, 50 μm). (**B**) Labeled neurons in paraventricular nucleus (compare with HYP in Fig. 2A) (scale bar, 100 μm). (**C**) Photomicrograph of brainstem section (compare with Fig. 2D). Dense labeling can be seen in locus coeruleus and subcoeruleus, raphe, and ventrolateral tegmentum contralateral to injections (scale bar, 600 μm).

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tered in the hindlimb area (*9, 19*), that is, in more medial and caudal locations than those observed after ulnar and median injections. In the red nucleus, the labeled neurons were concentrated in the ventral part, projecting to lumbosacral cord (*10*). Positive neurons were also found in the hypothalamus and at the multiple brainstem sites, as after ulnar and median injections.

The finding that the labeled neurons in the brain were restricted to the areas of origin of descending pathways indicates that they received virus by retrograde axonal transport after virus had been transferred from the peripheral nerves to terminals of these pathways in the spinal cord. An earlier study reported labeling of neurons in some of these brainstem sites after HSV-1 injection into sciatic nerve (*22*). However, this study regarded the brainstem labeling as "identical to that after intravenous inoculation and probably resulting from leakage of the inoculum into the blood stream" (*22*, pp. 192–193).

The differences in the distribution of labeled neurons in sensorimotor cortex and red nucleus after forelimb and hindlimb nerve injections indicate that virus was transferred only to terminals of those descending fibers that terminate in the spinal segments to which the injected nerves are connected. These nerves contain motor and sensory fibers that transported virus to the cord (Fig. 3). Therefore, the descending pathways may have received virus from retrogradely affected motoneurons as well as from incoming sensory fibers, which are distributed to ventral horn, intermediate zone, and especially to the dorsal horn. The sources from which the various descending pathways received virus must have been determined by their termination areas in the spinal gray matter. Thus, the neurons that project from the locus coeruleus and subcoeruleus, caudal raphe, and ventral reticular formation to the ventral horn (*14, 16, 23*) probably received virus either from affected motoneurons or from terminals of sensory fibers in the ventral horn or from both. The neurons that project from sensory cortex, hypothalamic paraventricular nucleus, Edinger-Westphal nucleus, ventrolateral pontine tegmentum, and raphe magnus to the dorsal horn, especially its outer laminae (*23–26*), presumably received virus from sensory fibers, either directly through axo-axonal contacts between sensory fibers and the fibers of the descending pathways, or indirectly via intercalated cells. Neurons in hypothalamic paraventricular nucleus and raphe nuclei may also have received virus from the autonomic lateral horn (*23, 24*). The finding that virtually no neurons in the medial pontine reticular formation (pars oralis) (Fig. 2, C and D) received virus was probably due to absence of virus in lamina VIII (Fig. 3), to which this pontine area mainly projects (*13*).

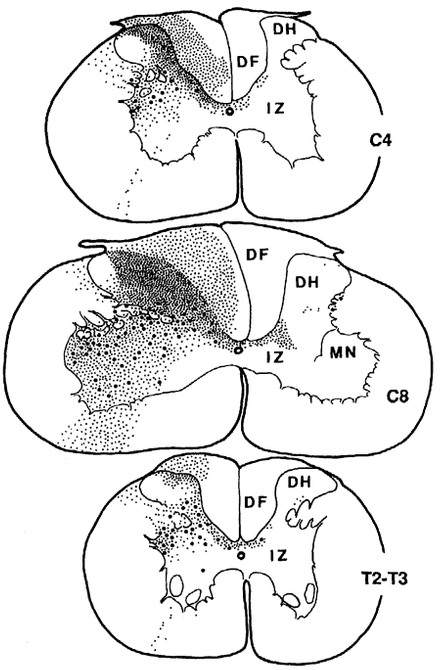

Fig. 3. Distribution of virus antigen (small dots) in the cervical and thoracic segments of the spinal cord after unilateral HSV-1 injections (left) into ulnar and median nerves. Large dots, large positive neurons. Abbreviations: DF, dorsal funiculus; DH, dorsal horn; IZ, intermediate zone; and MN, motoneurons.

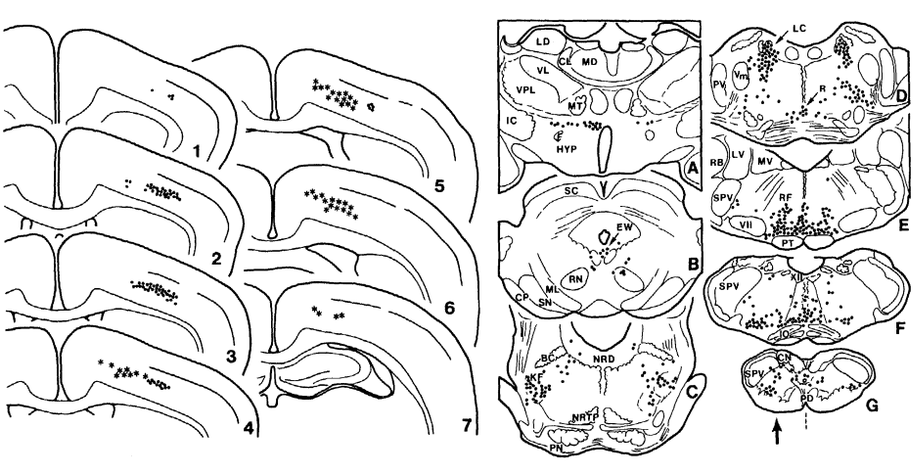

Fig. 2. (**1** to **7**) Distribution of HSV-1–labeled neurons in layer V of sensorimotor cortex contralateral to nerve injections. Lines represent densely granular layer IV observed in adjacent Nissl-stained sections. Dots in levels 1 to 5 represent neurons labeled after ulnar and median nerve injections. Asterisks in levels 4 to 7 represent neurons labeled after tibial nerve injections. Neurons labeled after ulnar and median injections are located lateral to neurons labeled after tibial injection. (**A** to **G**) Distribution of brainstem neurons (dots) labeled by HSV-1 after ulnar and median nerve injections (arrow, side of injection; sections are shown as A, rostral, to G, caudal). Abbreviations: BC, brachium conjunctivum; CL, nucleus centralis lateralis; CN, cuneate nucleus; CP, cerebral peduncle; EW, nucleus of Edinger-Westphal; F, fornix; HYP, hypothalamus; IC, internal capsule; IO, inferior olive; KF, nucleus of Kölliker-Fuse; LC, locus coeruleus; LD, nucleus lateralis dorsalis; LV, lateral vestibular nucleus; MD, nucleus mediodorsalis; ML, medial lemniscus; MT, mammillothalamic tract; MV, medial vestibular nucleus; NRD, nucleus raphe dorsalis; NRTP, nucleus reticularis tegmenti pontis; PD, pyramidal decussation; PN, pontine nuclei; PT, pyramidal tract; PV, nucleus principalis of the trigeminus; R, raphe nuclei; RB, restiform body; RF, reticular formation; RN, red nucleus; SC, superior colliculus; SN, substantia nigra; SPV, spinal trigeminal nucleus; VII, facial nucleus; VL, nucleus ventralis lateralis; VPL, nucleus ventralis posterolateralis; V_m, motor trigeminal nucleus; and XII, hypoglossal nucleus.

At 3.5 days after ulnar and median injection, labeling of neurons of the descending pathways was not accompanied by labeling of neurons along the ascending sensory pathways; labeling of the sensory part of the cuneate nucleus occurred only at 4 days. This delay may be explained as follows. Transport of virus through the sensory fibers ascending from "affected" cells in dorsal root ganglia might have been slower than retrograde transport through descending fibers, the cell bodies of which were not yet "affected."

In conclusion, in rats, HSV-1 is transferred from peripheral nerves to cortical and brainstem neurons that give rise to the descending pathways. This transneuronal transfer takes place in the spinal segments to which the nerves are connected. Therefore, HSV-1 may serve as a transneuronal tracer which would allow the identification of the different colonies of neurons in the brain that are related transsynaptically to different nerves (or muscles). Furthermore, tracing HSV-1 through a well-defined system of fiber connections may help to clarify the mode of virus propagation in the brain and to determine the influence of antiviral drugs on this process.

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Rapid Redistribution of Auxin-Regulated RNAs During Gravitropism

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Gravitropism, the bending of plants in response to gravity, is caused by differential growth rates on two sides of a responding organ. The general belief, although somewhat controversial, is that auxins play a major role in gravitropism by controlling the rate of cell extension. The tissue print technique was used to ascertain the distribution of auxin-regulated RNAs during the gravitropic response of soybean hypocotyls. In vertically oriented seedlings, auxin-regulated RNAs are symmetrically distributed in the elongating region of the hypocotyl. In horizontally orientated seedlings the distribution becomes asymmetrical within 20 minutes and the greatest asymmetry coincides with the onset of rapid bending. The results provide a clear correlation between the dynamic expression of genes under auxin control and a morphogenetic phenomenon traditionally known as an auxin response.

GRAVITY IS AMONG THE MOST IMportant determinants of plant growth. The gravitropic response results in the familiar pattern of plant growth (that is, aerial portions grow up and roots grow down). This differential growth response is thought to be mediated by the plant hormone auxin, since there is a large body of evidence that connects auxin with plant cell extension. Several groups have demonstrated that exogenously supplied auxin causes the accumulation of specific RNAs concomitant with (*1–3*) or prior to (*4*) auxin-induced cell extension. These studies suggest that at least part of the growth-controlling action of auxin is mediated by auxin-regulated gene expression. We have characterized a family of auxin-regulated RNAs from soybean that begin to accumulate within 2.5 min after application of auxin (*4*). We report here that gravistimulation rapidly alters the distribution of these RNAs in responsive organs and that the presence of the RNAs is highly correlated with cell extension. These results suggest that auxin-regulated gene expression is involved in the response of plants to gravity.

In excised elongating hypocotyl sections (EHSs), application of exogenous auxin results in a two- to fourfold enhancement of the rate of cell extension after a lag of about 15 min (*5*). We used the soybean hypocotyl system to identify cloned cDNAs corresponding to RNA species that rapidly and specifically accumulate in response to auxin (*3, 4*). Among these RNAs are a group of small auxin up RNAs (SAURs) that accumulate very rapidly after application of exogenous auxin to excised EHS (half-maximal accumulation after about 10 min; steady-state induction of 18- to 48-fold over controls within 30 to 60 min after application of 50 μ*M* 2,4-dichlorophenoxyacetic acid). The SAURs consist of three closely related families of RNAs of about 550 nucleotides. The 6, 10A, and 15 families show essentially identical RNA induction kinetics and auxin specificity with only subtle differences in other response parameters (*4*). Five SAUR genes have recently been sequenced, and at least three of the genes are transcriptionally regulated in the EHS (*6*).

We used the tissue print technique (*7*) modified for the detection of RNAs with ^{35}S-labeled antisense RNA probes to investigate the distribution of the SAURs during gravitropism. To increase the autoradiographic signal, we used a mixed SAUR probe (*8*) that contains four cDNAs. This probe detects only auxin inducible RNAs, whereas the RNA detected by the control clone [clone 5A (*9*)] is not auxin inducible (Fig. 1).

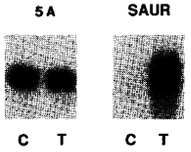

Fig. 1. Auxin inducibility of RNAs. RNA blot (*13*) of total RNA extracted from excised soybean EHS incubated for 4 hours (C) in the absence of auxin or (T) in the presence of 2,4-dichlorophenoxyacetic acid (50 μ*M*) for an additional hour. RNAs were detected with ^{32}P-labeled antisense RNA probes from cDNA clone 5A (*9*) or with a mixture of four SAUR cDNAs (*8*) as shown. RNA 5A consists of approximately 450 bases. The doublet band detected by the mixed SAUR probe consists of the 560-base RNAs hybridizing to the clone 6 family and the 530-base RNAs hybridizing to both the clone 10A and 15 families (*4*). Clone 6 family probes also detect a 1100-base auxin-inducible RNA (*4*) that is not visible in this exposure. Plant materials, growth conditions, RNA extraction procedures, and blotting methods have been described (*4*).

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