CNS Innervation of the Urinary Bladder Demonstrated by Immunohistochemical Study for c-Fos and Pseudorabies Virus

The aim of the present study is to verify the functional and anatomical neural pathways which innervate the urinary bladder in the central nervous system of the rat. To identify the functional neural pathway, the urinary bladder was stimulated by infusing formalin for 2 h. Then, brain and spinal cord were dissected out and immunohistochemistry was done by using anti-c-fos antibody. Many c-fos immunoreactive (IR) neurons were identified in the telencephalic cortical areas and in several brainstem nuclei, which are known mostly to be related with urinary bladder. In the spinal cord, a number of c-fos IR neurons were found in the lamina I, IIo, dorsal gray commissure, sacral parasympathetic nucleus. To identify the anatomical neural pathway of the urinary bladder, Pseudorabies virus (PRV) was injected into the wall of urinary bladder and was identified with anti-PRV by using immunohistochemistry. Most PRV labeled neurons were found where c-fos IR neurons were identified and few of them were also in the areas where c-fos IR neurons were not found, e.g., prefrontal cortex, agranular insular cortex, and subfornical organ. In the spinal cord, PRV labeled cells were found all over the gray matter. The present study presents morphological evidence demonstrating the supraspinal areas are related with the neural control of the urinary bladder and most functional neural pathway of the urinary bladder is well consistent with the anatomical neural pathway except in some telencephalic cortical areas. *(JKMS 1997; 12: 340~52)*

Key Words : *c-Fos, Pseudorabies virus, Neural axis, Urinary bladder, Spinal cord, Brain stem, Brain*

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Received : January 6, 1997
Accepted : April 21, 1997

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INTRODUCTION

The modulation of micturition is controlled in several levels of the central nervous system (CNS), including the lumbosacral spinal cord, rostral pons, hypothalamus, and the cerebral cortex (1). Various evidence indicates that the excitatory parasympathetic component of the micturition reflex is dependent upon the supraspinal pathway originating in an area of rostral pons the pontine micturition center (PMC) with descending fibers to the sacral cord. Damage to the central nervous system caudal to this pontine area causes profound urinary retention. In addition, the reflexes involved in urine storage are controlled by the sympathetic input to the smooth muscle of the bladder and internal urethral sphincter, and the somatomotor input to the external urethral sphincter are also modulated or coordinated by supraspinal centers in the rostral pons (2, 3). Also, there are important forebrain areas, especially from the hypothalamus and cortex that can modify these basic reflex pathways. Clinical studies have also shown that damage to potine and suprapontine structures whether due to trauma or disease of the central nervous system can cause, in some instances, a hyperactive bladder with urgency and incontinence and in other instances a hypo-active bladder with urinary retention. A detailed analysis of the neural circuit on the sacral parasympathetic nuclei has been the subject of past and ongoing investigations. However, the role and location of supraspinal, especially suprapotine structures, in control and modulation of micturition has been neglected.

It has been known that c-fos acts as a third messenger in signal transduction pathway and provides neurons with the ability to translate extracellular stimulation into long-term adaptive cellular responses by activation of genetic events (4~7). Additionally, the expression of c-fos is thought to be activated transsynaptically by peripheral stimuli in the CNS. Hence, it has been used to map metabolic activity of the activated neuronal cells in the CNS after various kinds of peripheral stimulation and generally shows a good correlation with known anatomical pathways. In the present study, distribution of c-fos immunoreactive (IR) neurons was assessed in the

CNS following chemical and mechanical stimulation of the urinary bladder to verify the functional neural pathway which controls the urinary bladder.

Pseudorabies virus (PRV) has been known that it is an ideal viral neural tracer for neuroanatomical studies because it has been shown to be transported in a retrograde transneuronal fashion in rats (8). In this study, we used an attenuated strain of the PRV - Barthas K strain - to study the general pattern of the central organization of the cell groups in the brain and spinal cord that regulate the urinary bladder function.

The present study attempted to verify the CNS cell groups that innervate the urinary bladder of the rats by using a transneuronal viral cell body labelling technique and c-fos immunohistochemistry and whether there is any difference between the functional neural pathway and the anatomical neural pathway of the urinary bladder in the CNS.

MATERIALS AND METHODS

Experimental procedure for induction of c-fos protein synthesis

Animal preparation

It is well known that c-fos gene can be induced by sensory stimuli other than those intentionally delivered by the investigator, care was taken to minimize uncontrolled variables. Therefore, rats were conditioned for our experiments; they were taken from their cages at the same time of the day and returned to their cage everyday for 1 week. Twelve female Sprague-Dawley rats were used in this study and anesthetized with urethane (1.2 mg/kg, i.p.). During the experiment, rats were placed prone in a comfortable position upon a soft towel, and both temperature of room and level of noise were standardized. Two animal groups were used in the analysis. One group (n=6) was for chemical irritation and mechanical stimulation to the urinary bladder. The other was (n=6) for control experiment.

The urinary bladder was catheterized through the urethra to infuse fluid and urethral outlet remained open. Chemical irritant (5% formalin in physiological saline) was infused into the bladder (0.12 ml/min) for 2 hours. When the urethra was open, the infusion solution could leak out or be expelled during a bladder contraction. Control experiments were also conducted in animals with a urethral catheter only.

Immunohistochemistry for c-fos protein

Rats were anesthetized with urethane (mg/kg) and perfused with 4% paraformaldehyde and 0.1% picric acid solution through aorta. Brain and L6/S1 segments of spinal cord were collected and postfixed in the same solution overnight. These were cryoprotected by placing them in 30% sucrose solution overnight at 4℃. Brain and spinal cord were sectioned with freezing microtome in 30 μm thickness.

The sections to be cut were incubated with anti-sheep anti-serum raised against c-fos diluted 1 : 8,000 in 1% normal rabbit serum in 0.05 M phosphate buffered saline (PBS) containing 0.1% Triton-X 100. This was followed by rinsing in PBS three times, ten minutes each, incubation with biotinylated goat anti-sheep IgG (Vector Lab, 1 : 200), rinsing in PBS, three times, ten minutes each, and incubated in the avidin-biotin complex solution (Vector Lab, 1 : 50). Avidin-biotin complexes were localized by reaction with a solution of 0.003% H_2O_2 and 0.05% diaminobenzidine tetrahydrochloride (DAB) in 0.1M phosphate buffer (PB). After the reaction the tissue sections were rinsed in the PBS and tap water and mounted on the gelatin-coated slides. They were dehydrated through low concentration to absolute ethanol and cleared in xylene. Slides were covered with cover glass and examined with a Nikon-FXA bright field microscope. They were drawn with drawing attachment on microscope. Numbers of c-fos IR cell in each brain area were counted on the drawings. They were marked on the schematic drawings of the brain.

Experimental procedure for PRV injection group

Animal preparation

Six adult female Sprague-Dawley rats were employed in the experiments. Animals were anesthetized with an intramuscular injection of Ketamine (85 mg/kg) and Xylazine (12 mg/kg) prior to aseptic surgery, which was carried out with the aid of a dissecting microscope. Care was taken to isolate the structure to be injected to prevent injection from spreading to adjacent structures. A mid-abdominal incision was made to expose the urinary bladder. Following retraction of the urinary bladder, it was gently lifted and placed on fresh gauze pads prior injection. Injection was confined to the fundus, body and neck. Following injection, the injection site was lightly rinsed with saline and spillage was mopped with gauze. These precautions, as well as isolating the injected structure with fresh gauze during the injection, were done to reduce tracer leakage to adjacent areas.

After a 3-days survival period, the rats were reanesthetized and perfused transcardially with heparinized PBS, followed by a periodate- lysine-paraformaldehyde fixative (PLP) (9). The brain and L6/S1 segments of spinal cord were removed, postfixed in PLP for 3 hours at 4℃, and then cryoprotected by placing them in 30%

sucrose solution overnight at 4℃.

Immunohistochemical localizations of PRV

Pseudorabies virus (Bartha strain) was used, grown in pig kidney fibroblasts to titers of 5×10^8 pfu/ml. A rabbit polyclonal anti-serum raised against PRV and the viral epitopes were localized with immunohistochemistry according to avidin-biotin modification (10).

Brain and spinal cord were sectioned with freezing microtome in 30 μm thickness. The sections to be cut were incubated with antirabbit antiserum raised against PRV viral epitope diluted 1:10,000 in 1% normal goat serum in 0.05 M PBS containing 0.1% Triton-X 100. This was followed by rinsing in PBS three times, ten minutes each, incubation with biotinylated goat anti-rabbit IgG (Vector Lab, 1: 200), rinsing in PBS, three times, ten minutes each, and incubated in the avidin-biotin complex solution (Vector Lab, 1:50). Avidin-biotin complexes were localized by reaction with a solution of 0.003% H_2O_2 and 0.05% DAB in 0.1 M PB. After the reaction, the tissue sections were rinsed in the PBS and tap water and mounted on the gelatin-coated slides. They were dehydrated through low concentration to absolute ethanol and cleared in xylene. Slides were covered with cover glass and examined with a Nikon-FXA bright field microscope. They were drawn with drawing attachment on the microscope. The numbers of PRV labelled cell in each brain area were counted on the drawings. They were marked on the schematic drawings of the brain.

RESULTS

Expression of c-fos protein

When urinary bladder catheterized rats were used as an experimental groups, it is possible to estimate that the number of neurons attributable to the chemical irritation of the bladder was obtained by subtracting the number of neurons induced by urethral catheter from the total number of c-fos expressed neurons following chemical irritation of the urinary bladder (Fig. 2a-r, 5a-f). For this reason, we are going to describe the locations which showed significantly increased number of c-fos expressed neurons after subtracting the number of c-fos expressed neurons following urinary catheter insertion alone (Fig. 3a-r, 6a-e) in that area.

In the brain stem, a cluster of c-fos IR neurons was observed in the middle and caudal nucleus tractus solitarius (Fig. 2p and q). These cells were located in the medial, intermediate, dorsal, and commissure subnucleus of the nucleus tractus solitarius. A significant number of c-fos IR cells were found in the ventrolateral medulla, bilaterally (Fig. 2p). In the Kölliker-Fuse nucleus and dorsal and dorsolateral nucleus of parabrachial nucleus, numbers of c-fos IR neurons were seen, bilaterally. In the midbrain, numbers of c-fos IR neurons were found

Abbreviations used in figures

AcbC	accumbens nucleus	LS	lateral septal nucleus
AI	agranular insular cortex	LV	lateral ventricle
AO	anterior olfactory nucleus	NSR	substantia nigra
AP	area postrema	ox	optic chiasm
Aq	aqueduct	PAG	periaqueductal gray
Bar	Barringtons nucleus	PB	parabrachial nuclei
CC	central canal	PC	paracentral thalamic nucleus
cp	cerebral peduncle	Pn	pontine nuclei
CPu	caudate putamen	PV	paraventricular thalamic nucleus
DEn	dorsal endopiriform nucleus	Py	pyramidal tract
DH	dorsal horn	R	red nucleus
DM	dorsomedial hypothalamic nucleus	SC	superior colliculus
DR	dorsal raphe nucleus	SO	supraoptic nucleus
EW	Edinger-Westphal nucleus	Sol	nucleus of the solitary tract
gcc	gunu of the corpus callosum	SV	spinal trigeminal nucleus
GP	globus pallidus	sV	spinal trigeminal tract
H	hippocampus	VH	ventral horn
IC	inferior colliculus	vhc	ventral hippocampal commissure
ic	internal capsule	VL	ventrolateral thalamic nucleus
IML	intermediolateral cell column	VMH	ventromedial hypothalamic nucleus
LC	locus coeruleus	VPL	ventral posterolateral thalamic nucleus
LH	lateral hypothalamic area	VPM	ventral posteromedeal thalamic nucleus
LPGi	lateral paragigantocellular nucleus	III	3rd ventricle
LPO	lateral preoptic area		

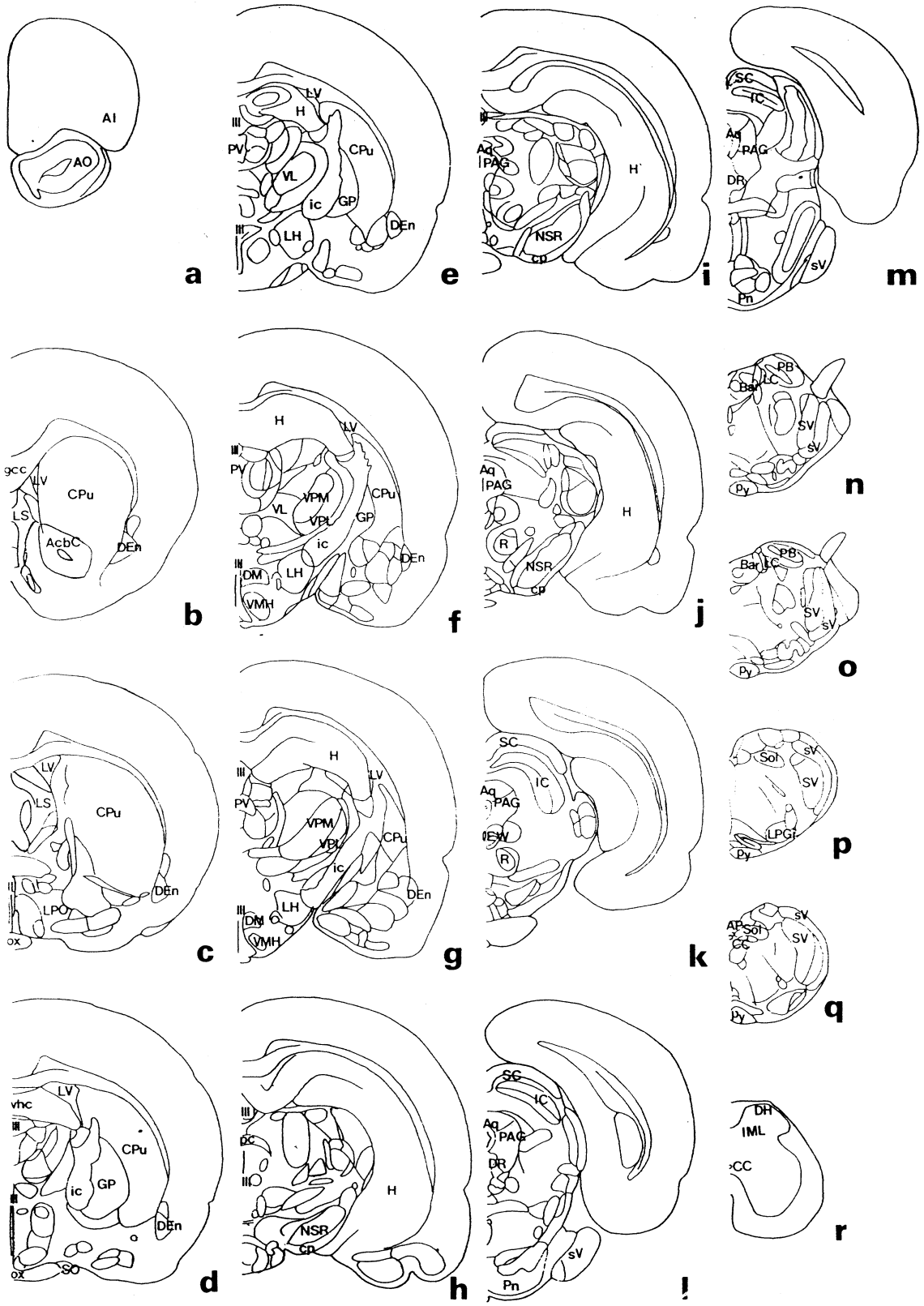

Fig. 1. Line drawings showing the areas of the central nervous system of the rat. The level of the section was determined intentionally. Arranged from rostral (a) to caudal (q) in the frontal plane. Reference level: interaural line (0.0 mm). Drawings were modified from Paxinos and Watson (31).

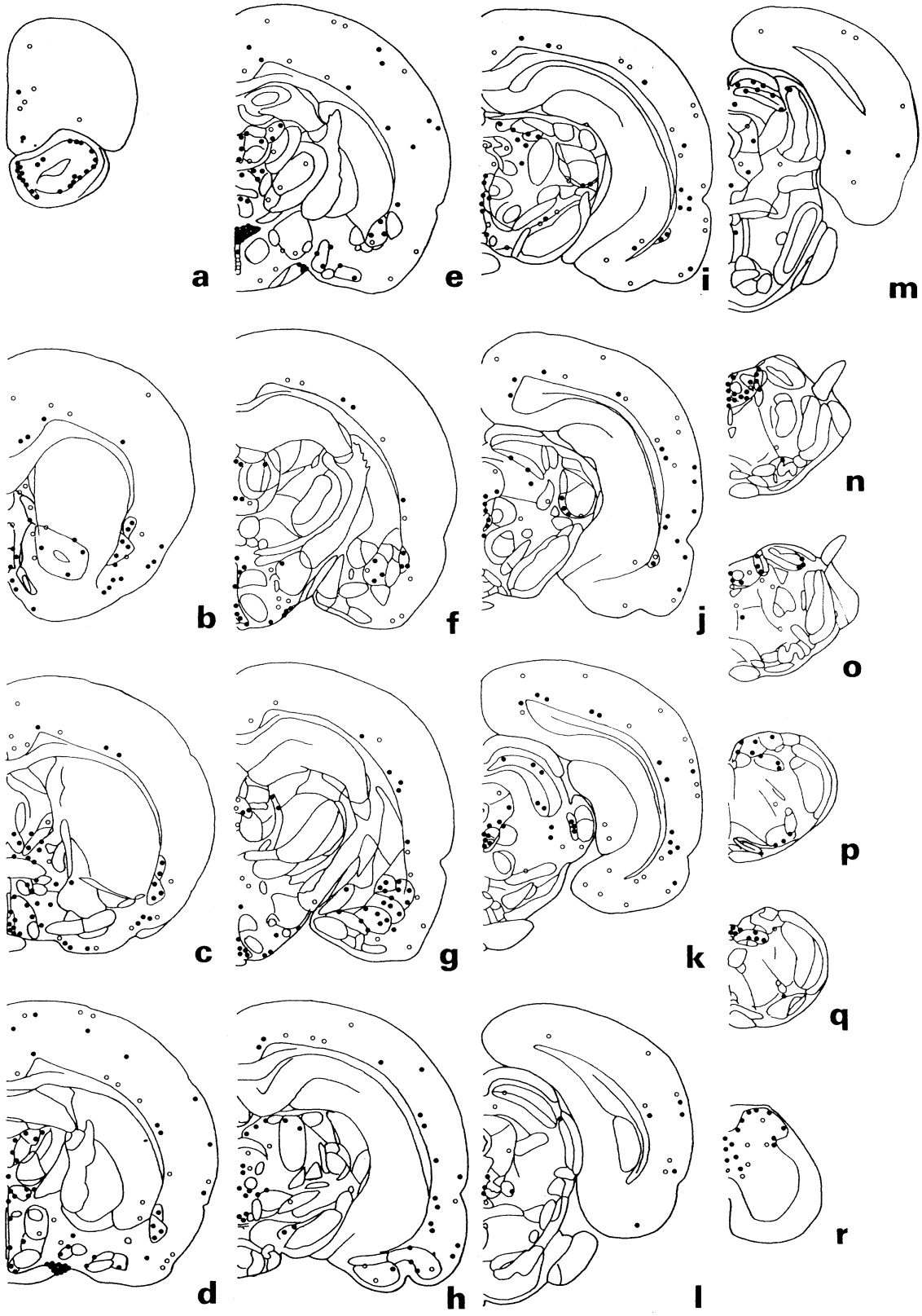

Fig. 2. Schematic drawings showing the distribution of c-fos immunoreactive neurons in the rat brain and spinal cord after formalin infusion into the urinary bladder. Closed dot (●) indicates an immunoreactive cell group which consists of 5 cells. Open dot (○) indicates an immunoreactive cell group containing 2 cells.

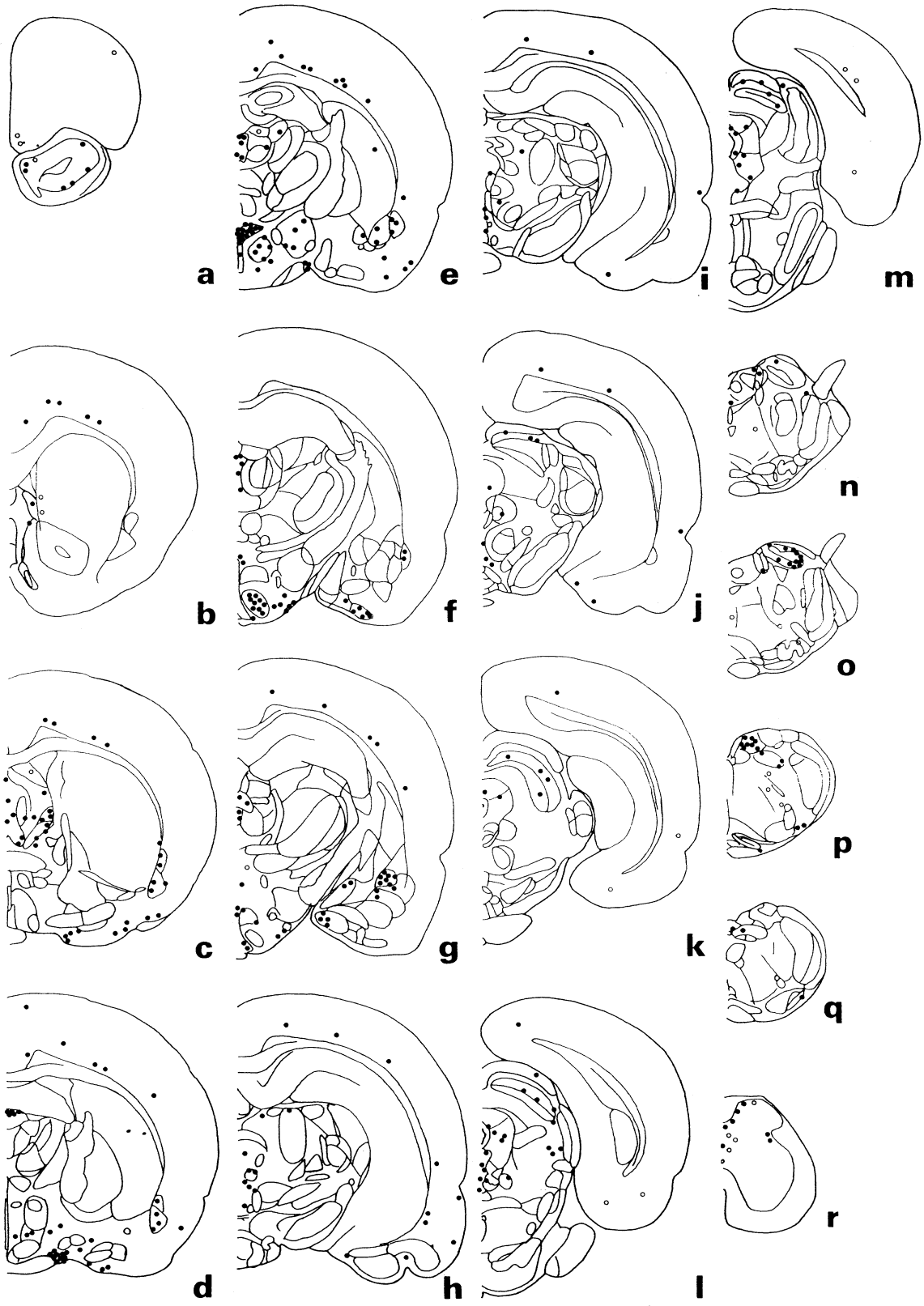

Fig. 3. Schematic drawings showing the distribution of c-fos immunoreactive neurons in the rat brain and spinal cord after insertion of catheter into the urinary bladder through the urethra. Closed dot (●) indicates an immunoreactive cell group which consists of 5 cells. Open dot (○) indicates an immunoreactive cell group containing 2 cells.

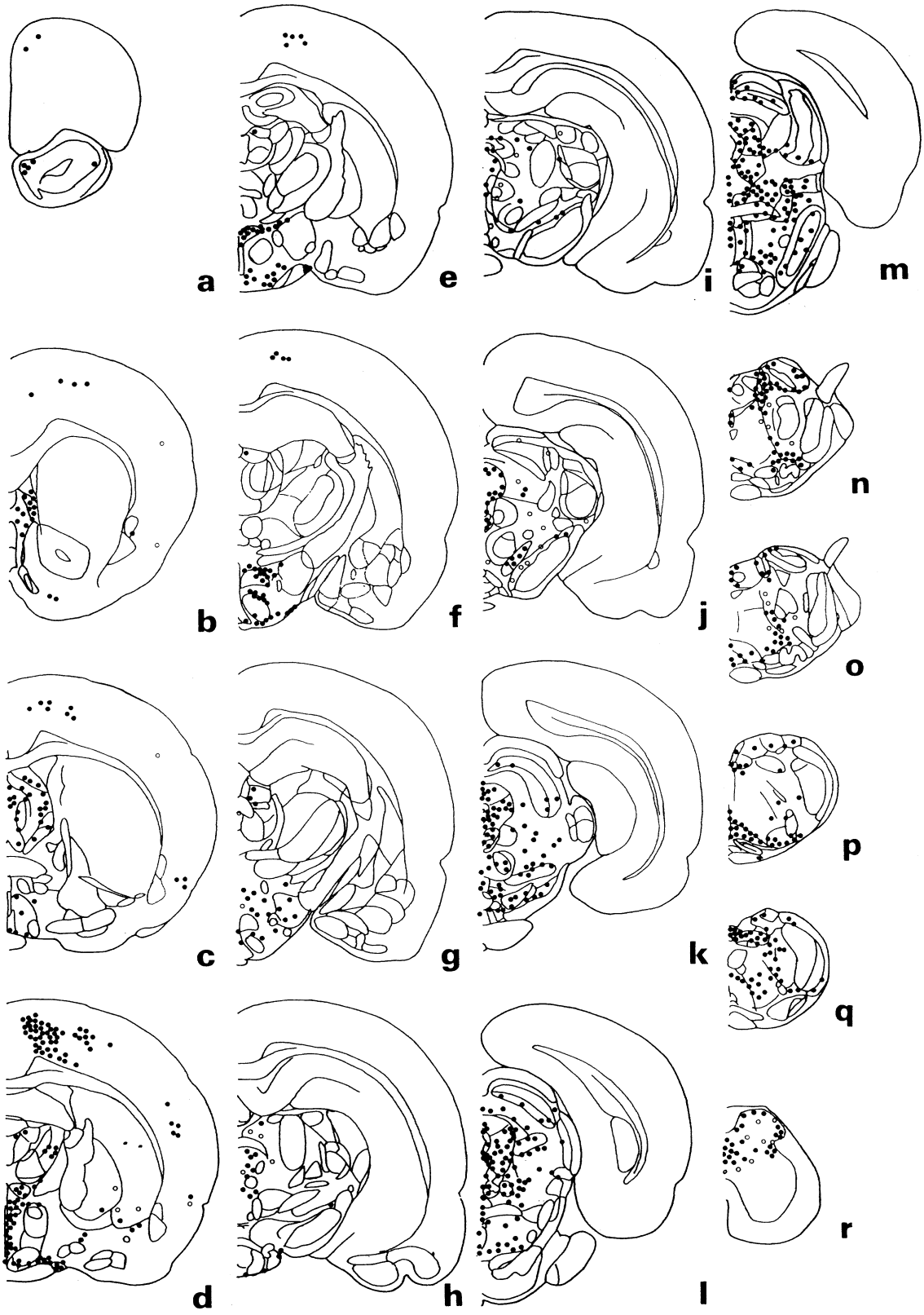

Fig. 4. Schematic drawings showing the distribution of the cell body labeling in the rat brain after a pseudorabies virus infection of the urinary bladder. Closed dot (●) indicates an immunoreactive cell group which consists of 5 cells. Open dot (○) indicates an immunoreactive cell group containing 2 cells.

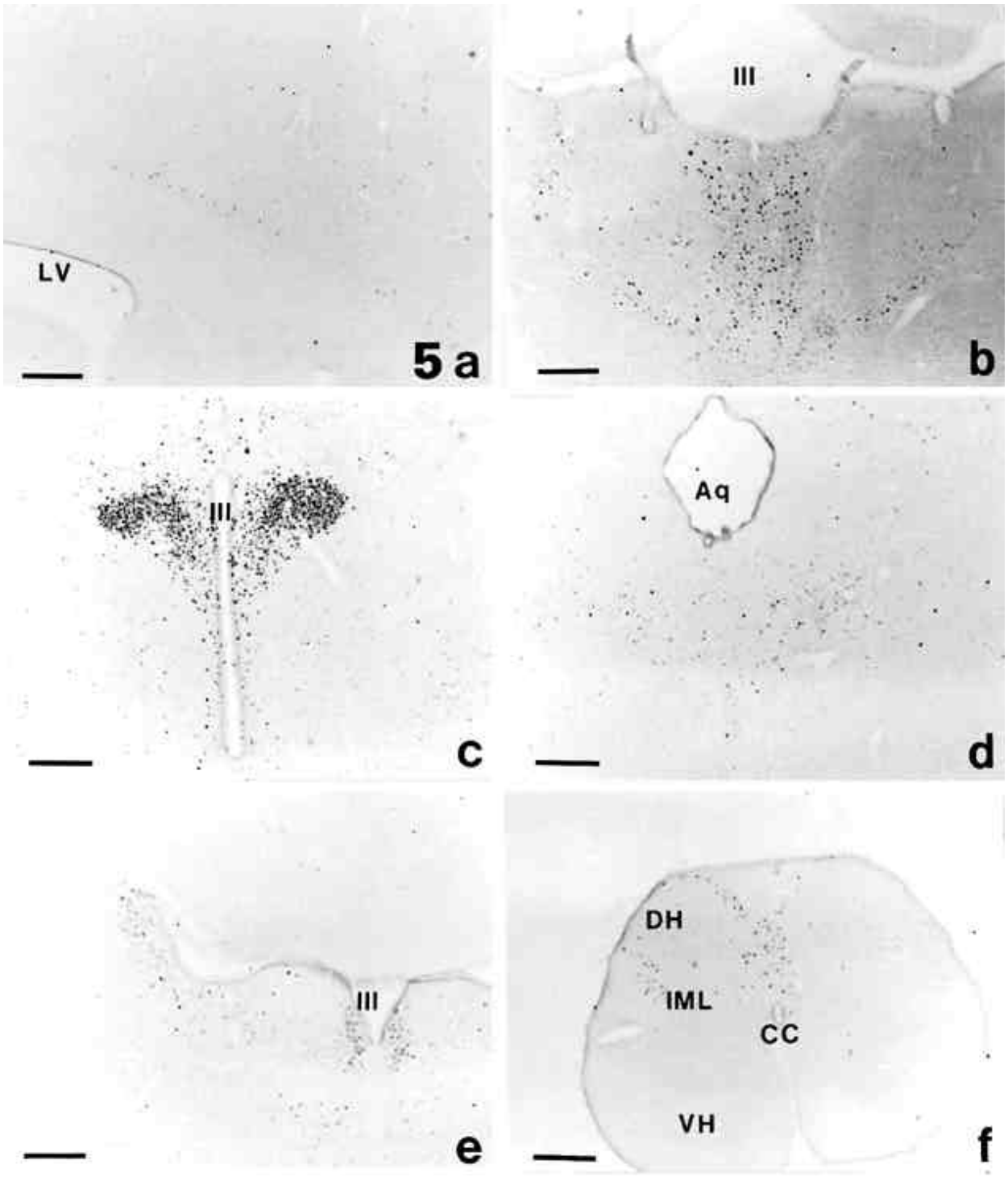

Fig. 5. C-fos immunoreactive neurons in the prefrontal cortex (a), the periventricular nucleus of the thalamus (b), the paraventricular hypothalamic area (c), the periaqueductal gray (d), the pontine micturition center (e), and the L6 spinal cord (f) after formalin infusion into the urinary bladder. Bars equal 250 μm.

in the dorsal raphe nucleus and ventrolateral nucleus of periaqueductal gray (Fig. 2i-o, 5d and e).

In the diencephalon, increased immunoreactivity was observed in the ventromedial nucleus, dorsomedial nucleus, medial tuberal nucleus, arcuate nucleus, periventricular nucleus (Fig. 5c) and lateral hypothalamic area of the hypothalamus (Fig. 2d-g). Numbers of c-fos IR cells were expressed in the paraventricular nucleus (Fig. 5b),

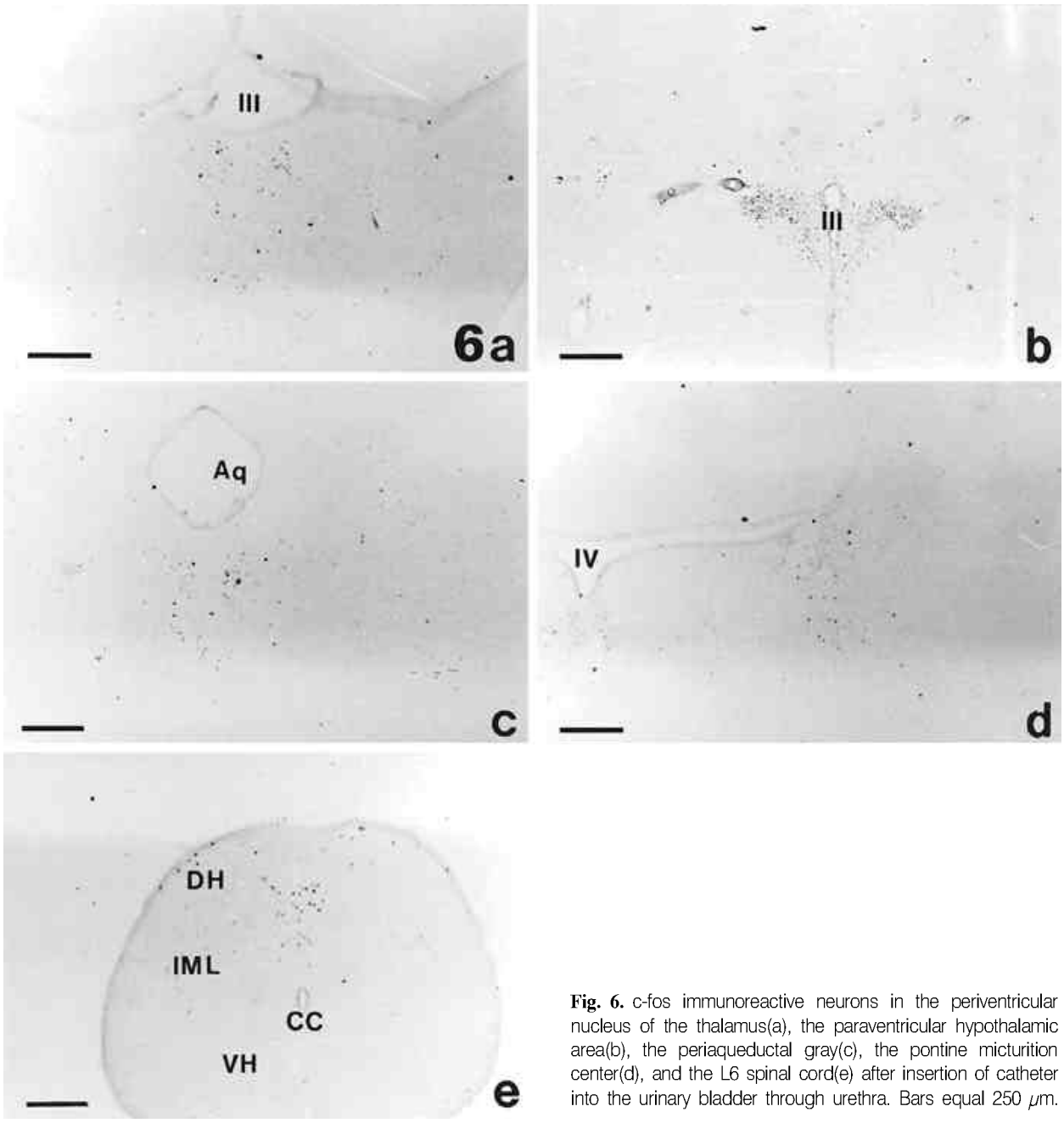

Fig. 6. c-fos immunoreactive neurons in the periventricular nucleus of the thalamus(a), the paraventricular hypothalamic area(b), the periaqueductal gray(c), the pontine micturition center(d), and the L6 spinal cord(e) after insertion of catheter into the urinary bladder through urethra. Bars equal 250 μm.

central medial nucleus, and central lateral nucleus of the thalamus (Fig. 2d-h).

In the telencephalon, significantly increased numbers of c-fos IR cells were shown in the olfactory cortex, prefrontal cortex (Fig. 5a), and piriform cortex (Fig. 2a-j). Clusters of labelled cells were observed bilaterally in the amygdaloid nuclear complex following formalin stimulation (Fig. 2c-i) and in the catheter insertion group (Fig. 3c-i).

In the spinal cord, c-fos IR neurons were observed in the sacral parasympathetic nucleus, dorsal gray commissure and around central canal of the experimental groups (Fig. 5f). Numbers of c-fos IR neurons in these areas were significantly increased following formalin stimulation (Fig. 2r and Fig. 5f) compared to the catheter insertion group (Fig. 3r and 6e).

PRV labelled cell groups

In the spinal cord, PRV labelled cells were found throughout the gray matter, concentrated in the intermediolateral nucleus, around central canal, dorsal gray commissure and dorsal horn (Fig. 4r and 7f).

In the brain stem, a number of PRV labelled cells were

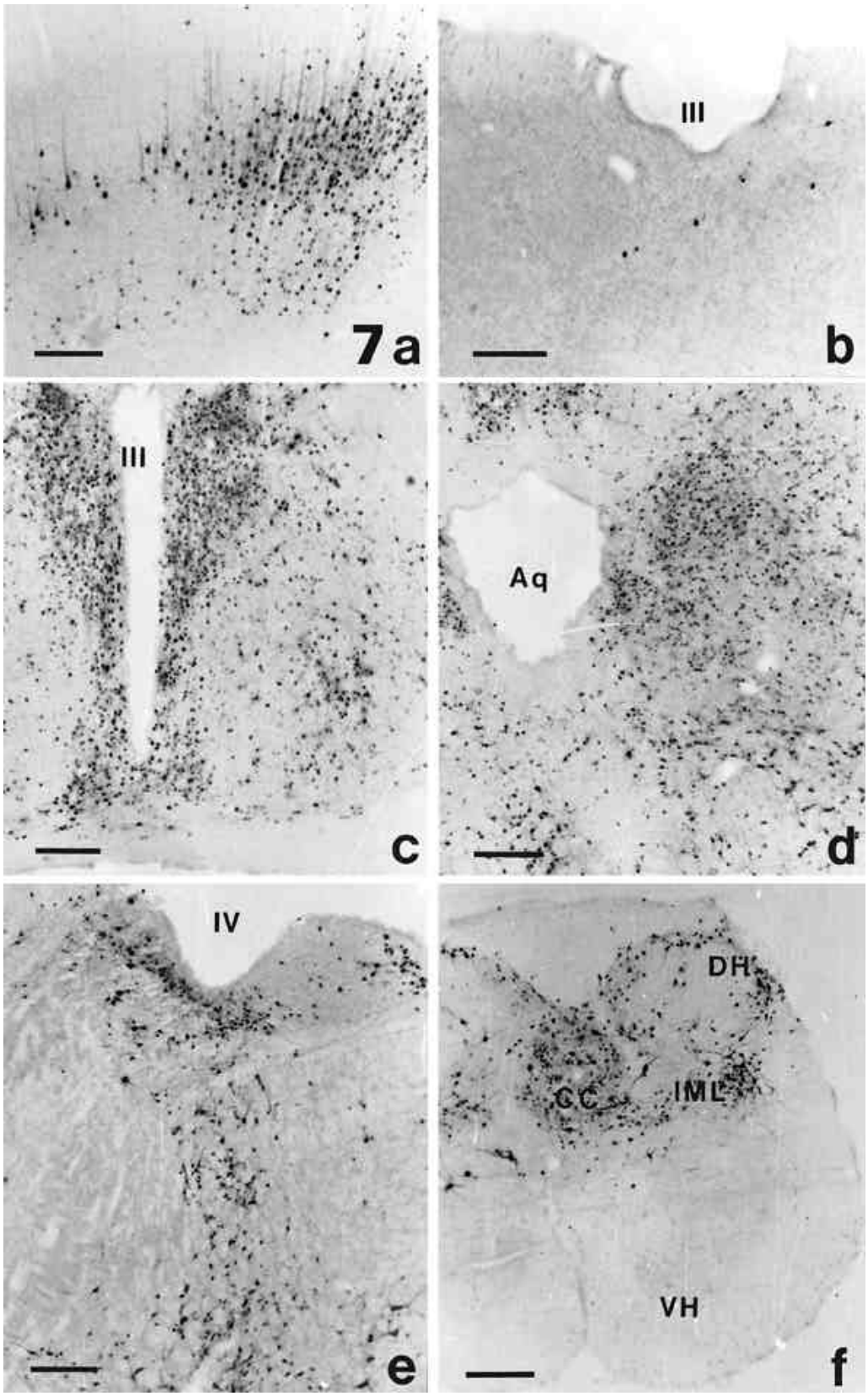

Fig. 7. Transneuronal cell body labeling in the prefrontal cortex (a), the periventricular nucleus of the thalamus (b), the paraventricular hypothalamic area (c), the periaqueductal gray (d), the pontine micturition center (e), and the L6 spinal cord (f) after a pseudorabies viral infection of the urinary bladder. Bars equal 250 μm.

seen in the A5 noradrenergic cells, raphe magnus, raphe obsculus, raphe pallidus, gigantcelluraris reticularis, area postrema, nucleus tractus solitarius, substantia nigra pars compacta, red nucleus, Edinger-Westphal nucleus, ventral, dorsolateral and ventrolateral subnucleus of the periaqueductal gray, dorsal raphe, locus ceruleus, Barringtons nucleus, and Kölliker-Fuse nucleus (Fig. 4i-q, 7d, and e).

In the diencephalon, PRV labelled cells were observed in the bed nucleus of stria terminalis, organum vasculosum of lamina terminalis, lateral hypothalamic area, paraventricular nucleus, retrochiasmatic nucleus, arcuate nucleus, suprachiasmatic nucleus, and median preoptic area (Fig. 4d-h, 7b, and c).

In the telencephalon, PRV labelled cells were seen in the agranular insular cortex, prefrontal cortex, subfornical organ, and septal nucleus (Fig. 4a-f, and 7a).

DISCUSSION

In decerebrate animals where the entire forebrain rostral to the superior colliculus has been removed, micturition behavior would be done reflexly, however, they lose their ability to modify their behavior with respect to time, place or emotional state (11). This is consistent with the idea that, although micturition itself is very simple reflex behavior, it also needs modulation to maintain social lives. The neural circuit of the micturition reflex may, therefore, need modulation sites and have connections throughout the CNS. Neuroanatomical, electrophysiological, and clinical studies have shown that among many areas of the CNS, certain areas of the cerebral cortex, the hypothalamus, and the midbrain can modify or modulate the basic micturition reflex (1, 12, 13).

Electrophysiological data show that stimulation of various sites on the surface of the cortex have either excited or inhibited bladder functions in cats (14). Electrical stimulation of the cingulate gyrus shows excitatory and inhibitory effects on the bladder function. When the orbital cortex is electrically stimulated, bladder function is mostly inhibited. The sylvian gyri has mostly inhibitory with some excitatory sites for bladder activity. Motor cortex has mostly excitatory with a few inhibitory sites to bladder activity and ablation of the motor cortex causes a decrease in the capacity of the bladder in cats. Clinical reports in patients also support the experimental data in animals. For instance, patients with pathologic or surgical lesions to the superior frontal gyrus often have shown hyperactive bladders with urgency and incontinence. In our experimental group, c-fos expressed neurons are found in the prefrontal cortex, pyriform cortex, insular cortex and orbital cortex following chemical and mechanical stimulation of the urinary bladder. And PRV labeled cells are also observed in the prefrontal cortex and insular cortex following PRV injection into the urinary bladder. Our findings support the idea that motor cortex and sensory motor association cortex are involved in the modulation of micturition.

Among subcortical areas, the septal area and subcortical area are thought to modulate the micturition behavior. Electrical stimulation of the medial preoptic nucleus of the cat has been reported to evoke a micturition-like contractions (15). Furthermore, previous report applying electrical stimulation to the septal and preoptic nucleus produced a complete micturition behavior in awake, freely moving cats. Preoptic area projects directly to the micturition center (16, 17). These reports are well consistent with the findings that a significant number of c-fos expressed neurons and PRV labelled neurons are observed in the septal nucleus and preoptic nucleus of our experimental groups. Considering the fact that micturition reflex is not abolished by precollicular decerebration, these area are also involved in the modulation of micturition reflex.

Previous study report an increase in bladder tone following anterior hypothalamic stimulation and a decrease in tone following posterior hypothalamic stimulation (18). Lesion generating experiments also support the idea that inhibitory and facilitatory sites exist in the hypothalamus which modulate bladder activity (19, 20). Degenerating fibers following lesions to the hypothalamus could be traced to various brainstem areas including structures near the PMC (21). According to tract tracing study, there are direct projecting neurons to the hypothalamus in the sacral parasympathetic nucleus (22). And, the present study also shows that c-fos expressed cells were found in the some subnucleus of the hypothalamus, i.e., paraventricular nucleus, arcuate nucleus, supraoptic nucleus, lateral hypothalamic nucleus, and retrochiasmatic nucleus following chemical and mechanical stimulation. PRV labelled cells were also examined in the same subnucleus of the hypothalamus. These data suggest that the hypothalamus provides direct input to neurons within the PMC and also to autonomic centers in the sacral cord and may play a role in the modulation of micturition reflex.

Electrophysiological data have shown excitation of many neurons in the potine micturition center following stimulation of sacral parasympathetic nucleus. The pontine micturition center in the rat is known to be labelled following the injection of tracers into the intermediolateral area in the L6/S1 spinal cord and also causes bladder contractions when stimulated electrically or urine retention when ablated bilaterally. These experimental

results suggest that there are direct neural connections between the PMC and sacral autonomic nucleus.

According to studies using tracer, neurons in the dorsolateral tegmental nucleus have direct projections to the intermediolateral nucleus of the spinal cord. In the intermediolateral areas of the L6/S1 spinal cord, serotoninergic, dopaminergic, and noradrenergic (23) fibers, are found densely and these fibers are originated from cell groups which contain these neurotransmitters in the brain stem. These neuroanatomical and immunohistochemical studies have suggested that catecholaminergic area in the brainstem have direct projection into the intermediolateral areas in the L6S1 spinal cord (24). We found that many c-fos expressed cells and PRV labelled cells existed in the PMC, locus ceruleus, periaqueductal gray, dorsal raphe nucleus, Kölliker-Fuse nucleus, ventrolateral parabrachial nucleus, substantia nigra, and reticular formation.

A variety of data from these earlier experimental and clinical reports include almost all the regions which are the c-fos expressed neurons following chemical and mechanical stimulation of the urinary bladder and virus labeled neurons following PRV injection into the urinary bladder in our study. This means that most regions in our study are thought to be involved in the neural regulation of urinary bladder function.

The distribution pattern of c-fos immunoreactive neurons following noxious chemical and mechanical stimulation of urinary bladder was consistent with that of the PRV labelling neurons. However, there are some discrepancies between the c-fos expression pattern following chemical and mechanical stimulation of the urinary bladder and the distribution pattern of the PRV labelling neurons following PRV injecting into the urinary bladder. For example, c-fos was not expressed in the circumventricular organ such as subfornical organ and organum vasculosum of lamina terminalis where PRV labelling neurons were found. Instead, in the limbic cortex such as olfactory cortex, pyriform cortex, entorhinal cortex, dorsal endopyriform nucleus, amygdaloid nuclear complex, and in the periventricular nucleus of the thalamus, c-fos was expressed but PRV labelled neurons were not found. There are at least 3 possibilities for the discrepancy between distribution pattern of the c-fos expression after formalin irritation of the urinary bladder and PRV labelling pattern following PRV injection into the urinary bladder. First, according to previous report, the mesencephalic nucleus of the trigeminal nerve was not labeled with virus when the herpes virus was injected into the trigeminal nervous system (25) and the neurons in the olfactory pathway are not infected with virus after neurotrophic virus instillation into the nasal mucosa (26). These experimental data support the idea that not all the CNS areas related with the peripheral organ may be labeled with the viral tracer injected into them. Hence, all the CNS area have neural connections with the urinary bladder may not be labeled with PRV in our trial. Second, the fact that neurotrophic viruses are transported in a retrograde transneuronal fashion (25~28) suggests that the motor neural pathway is infected by viruses, earlier than the sensory neural pathway. Therefore, it can be speculated that the labeled neural pathway after injecting neurotrophic viruses is related with motor innervation of the infected organ in the earlier period. In addition, it is conceivable that most PRV labeled areas belong to the motor neural pathway of the urinary bladder in the present study. In contrast to PRV, all the CNS areas expressing c-fos may cover the sensory and motor component of the peripheral stimulated foci. Third, the reason why c-fos was expressed in unexpected areas is probably caused by uncontrollable experimental stress during 2 hours of formaline stimulation. It has been known that c-fos is easily induced with the various kind of stimuli (4, 6, 29, 30). Indeed, it is not easy to remove the unwanted stimuli completely in experiments although that we tried to restrict other stimuli except the very stimuli wanted during c-fos inducing experiment.

In summary, the results of the present study have presented morphological evidence indicating that, while it may differ in some telencephalic cortical areas, the functional neural pathway of the urinary bladder by inducing c-fos expression is well consistent with the anatomical neural pathway, in additional to the contention that many supraspinal as well as pontine micturition centers are involved in the neural control of the urinary bladder. It is, however, not certain that each area has actual synaptic connections with urinary bladder although many supraspinal areas have been proved to be involved in the functional and anatomical neural pathway of the urinary bladder in this study. The synaptic and functional connection among these areas by using neural tracers and electrophysiological approaches can be investigated on the basis of this study.

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