J Korean Med Sci 2001; 16: 209-13
ISSN 1011-8934

CNS Cholinergic Innervation to the Hippocampus in the Rat Using Pseudorabies Virus as a Neurotracer

The hippocampus is a central area of the memory-related neural system. Combined immunohistochemistry against choline acetyl transferase and retrograde transneuronal labelling of the pseudorabies virus were used to identify cholinergic neurons in the central nervous system projecting to the hippocampal formation of the rat. Five to ten μL of Bartha strain of pseudorabies virus were injected into the dentate gyrus, CA1 and CA3 of the hippocampus of 20 Sprague Dawley rats using stereotaxic instrument. Forty eight to 96 hr after the injection, the brains were removed and the tissue sections were processed for double immunofluorescence procedure using polyclonal antibodies against pseudorabies virus or choline acetyl transferase. The double labelled neurons were distributed at several different nuclei and the labelling patterns of three different areas of the hippocampus were similar. These data suggests that the cholinergic innervation to the hippocampus were distributed in a transsynaptic manner throughout the whole brain area.

Key Words: *Hippocampus; Pathway; Herpesvirus 1, Suid; Acetylcholine*

Mihee Ko, Moonyou Oh, Haesook Noh*, Moonjae Cho†, Youngjae Lee†, Bonghee Lee

Departments of Anatomy and Neurobiology, Biochemistry†, Medical School, Cheju National University; Department of Pharmacology†, Veterinary Medical School, Cheju National University, Cheju, Korea
Department of Anatomy and Neurobiology*, Medical School, Gyeongsang National University, Chinju, Korea

Received: 9 August 2000
Accepted: 24 November 2000

Address for correspondence
Bonghee Lee, DVM, Ph.D.
Department of Anatomy and Neurobiology, Medical School, Cheju National University, 1 Ara 1-dong, Cheju 690-756, Korea
Tel : +82.64-754-3822, Fax : +82.64-725-2593
E-mail : bhlee1@cheju.cheju.ac.kr

• This work was supported by grant No. 971-0707-055-2 and 1999-1-213-003-2 from the interdisciplinary Research program of the KOSEF.

INTRODUCTION

Alzheimer's disease is a progressive and neurodegenerative disease of the central nervous system (CNS) mainly characterized by the deterioration of memory function. The hippocampus is a central area of the memory-related neural system (1, 2), where it receives several axonal innervations from different area of the brain. Although the medial septum and the diagonal band of Broca have been known as major areas of cholinergic inputs to the hippocampus (3-5), the precise location of the whole cholinergic neurons in the central nervous system projecting to the hippocampal formation have remained elusive due to the lack of an appropriate transneuronal neurotracer and labelling method.

Since Martin and Dolivo (6) first presented the idea, viruses have been used as a transneuronal markers. The Bartha strain of pseudorabies virus has been widely used as a useful tool in the neuroanatomical studies (7-9). Recently, acetylcholine has been reported as a neurotransmitter implicating in a variety of behavioral process including memory function and choline acetyl transferase (ChAT), the synthesizing enzyme for acetylcholine (ACh), has been recognized as a definite marker for cholinergic neurons (10-13).

In this study, the transneuronal neurotracer, Bartha strain of the pseudorabies virus, was used in combination with the immunohistochemical procedures to identify and compare the distributions of the CNS cholinergic neurons projecting to the three different areas (dentate gyrus, CA1 and CA3) of the hippocampus.

MATERIALS AND METHODS

Adult 20 Sprague Dawley rats (230-350 g) were used in this study. Animals were anesthetized with ketamine HCl (0.75 mg/kg) and xylazine (1 mg/kg) prior to surgical procedures. Injection of pseudorabies virus was made into the dentate gyrus, CA1, and CA3 of the hippocampus with an aid of the stereotaxic instrument. Injection was carried out following the midline incision

of the scalp skin. The skull was pierced with biological electric drill at various points from bregma [dentate gyrus, 3.7 mm (AP), 2.4 mm (Lateral); CA1, 3.7 mm (AP), 2.7 mm (Lateral)]; CA3, 3.7 mm (AP), 3.6 mm (Lateral) and Hamilton syringe was lowered vertically until it reaches the injection areas (dentate gyrus, 3.2 mm; CA1, 2.6 mm; CA3, 3.6 mm). A total 5-10 μL of the pseudorabies virus was injected slowly at the speed of 1 μL per min. Then syringe was removed slowly and surgical wounds were sutured with wound clips. Most of the rats were allowed to survive for 2-4 days post-injection. All rats were reanesthetized in same manner and perfused transcardially with 100-200 mL of heparinized saline (18℃) followed by 400 mL of 4% paraformaldehyde -lysine periodate in 0.1 M sodium phosphate buffer (pH=7.4). The brains were removed, placed in the same fixative for 4 hr at 4℃, and then transferred to 20% sucrose/0.1 M phosphate buffer solution until sink fully at 4℃. The brains were cut in transverse plane at 30 μm on a freezing microtome.The free floating tissue sections were processed for double immunofluorescence procedure using polyclonal antibodies to pseudorabies virus or choline acetyl transferase. One of every six sections was incubated overnight at 38℃ with the mixture of rabbit anti-pseudorabies virus (1:100) and goat anti-choline acetyl transferase (1:20, Chemicon Int'l Inc., diluted in 0.1 M sodium phosphate buffer containing 1% normal donkey serum and 0.3% Triton X-100). After 24 hr, the sections were reacted for 2 hr with a cocktail of FITC-labelled donkey anti-rabbit IgG (1:50, Jackson Immunoresearch Lab.) and TRITC-labelled donkey anti-goat IgG (1:50, Jackson Immunoresearch Lab.). Thereafter, the sections were washed, mounted onto gelatin-coated slides and coverslipped with glycerol-PBS (29:1). Cells were counted as positive if a reasonable portion of the cell body was visible in the section.

RESULTS

Immunohistochemical localization of PRV-Ba and ChAT

After injection of pseudorabies virus (PRV-Ba) into the dentate gyrus, CA1 or CA3 area of the hippocampus and double immunofluorescent staining, both PRV-Ba and ChAT immuno-positive cells were observed in several different nuclei of the brain from the cerebrum to the brainstem in same manner (Table 1). Most of the positive neurons were multipolar in shape (Fig. 1).

Distribution of the PRV-Ba and ChAT positive cells

The PRV-Ba and ChAT positive cells were distributed differently in labelling intensity according to the nuclei. Among the positive nuclei, the medial septal nucleus, the nucleus of the diagonal band, the entorhinal cortex, the central amygdala, the lateral hypothalamic area and the bed nucleus of stria terminalis showed strong positive labelling (Table 1, Fig. 1). The agranular insular cortex, gigantocellular reticular nucleus showed weak labelling. The caudate putamen, the dorsal motor nucleus of vagus nerve, the insular cortex, the primary motor area, the medial preoptic nucleus, and the reticular nucleus showed moderate positive labelling to both PRV-Ba and ChAT. The labelling patterns of the dentate gyrus, CA1 and CA3 were similar through the whole brain area.

Table 1. Quantitative analysis of the double immunoreactive cells in the brain after PRV-Ba injection into the dentate gyrus CA1, or CA3 area of the hippocampus

Nucleus	Injection site		
	Dentate gyrus	CA1	CA3
Agranular insular cortex (Ald)	+	+	+
Bed nucleus of stria terminalis (BNST)	+++	+++	+++
Central amygdala (CeA)	+++	+++	+++
Caudate putamen (CP)	++	++	++
Dorsal motor nucleus of vagus nerve (DMV)	++	++	++
Entorhinal cortex (ENT)	+++	+++	+++
Gigantocellular reticular nucleus (GRN)	+	+	+
Insular cortex (ICj)	++	++	++
Lateral hypothalamic area (LHA)	+++	+++	+++
Primary motor area (MOp)	++	++	++
Medial preoptic nucleus (MPO)	++	++	++
Medial septal nucleus (MS)	+++	+++	+++
Nucleus of the diagonal band (NDB)	+++	+++	+++
Reticular nucleus (RTN)	++	++	++

Immunoreactivity: +, moderate; ++, intermediate; +++, strong

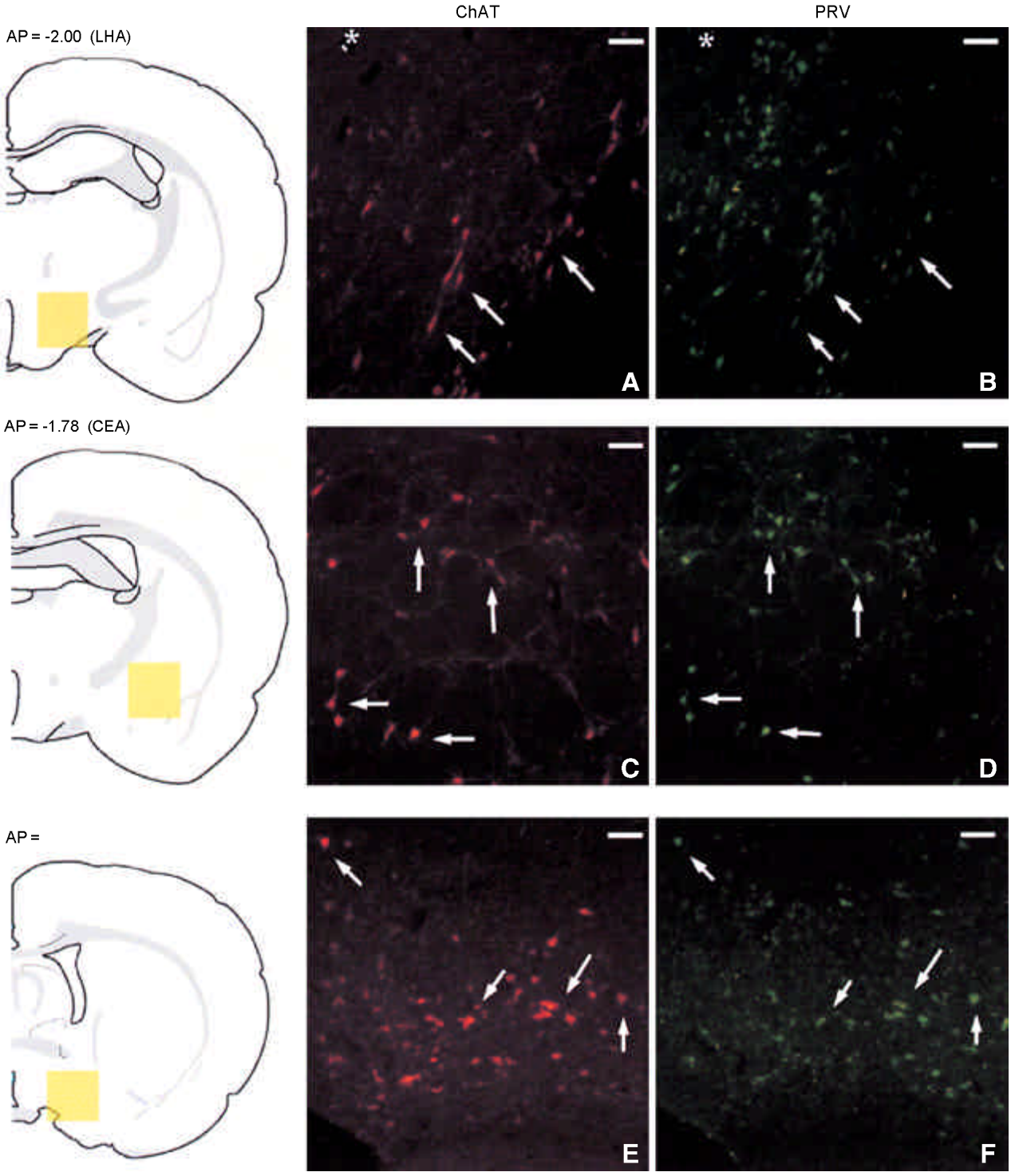

Fig. 1. Pseudorabies virus (PRV) (FITC) and choline acetyl transferase (ChAT) (TRITC)-double labelled cells in the different brain areas after PRV injection into the hippocampus of the rat. **A, B:** lateral hypothalamic area (LHA); **C, D:** central amygdala (CEA); **E, F:** nucleus of diagonal band (NDB); Bar=250 μm.

DISCUSSION

The hippocampus is widely believed to exert an important influence on memory. Several neuronal pathways including the septohippocampal system are thought to play a crucial role in the performance of a variety of complex learning and memory tasks (14, 15). In cognitive and memory processes, cholinergic involvement has

been known as one of the most important transmitter system. Previous studies have shown that PRV-Ba produces highly specific infections within a given group of synaptically connected neurons without spreading to adjacent, functionally unrelated neuronal networks (16-20). In as much as we have used this genetically attenuated strain of the virus, and not the wild type forms which can produce non-specific labelling (21), our results provide evidence for the longheld, but hitherto unsubstantiated, belief that certain subsets of central neurons are able to influence memory function via a neurally mediated mechanism.

In the present study, we examined the transsynaptic cholinergic circuitry projecting to the dentate gyrus, CA1 or CA3 of the hippocampus using the pseudorabies virus as a neurotracer. The location of the transsynaptically labelled neurons is, in general, consistent with earlier studies showing that lesions or stimulation of some brain regions affect memory function independent of the hippocampus.

Our results show that cholinergic innervations are localized in the medial septal nucleus, the nucleus of the diagonal band, the entorhinal cortex, which are known as three major cholinergic nuclei to the hippocampus (4, 5, 13). These results indicate that the pseudorabies virus could label the primary cholinergic nuclei innervating the hippocampus specifically. Additionally, the labellings were also appeared at the several nuclei including the central amygdala, the lateral hypothalamic area, and the bed nucleus of stria terminalis.

These results are also related to the tracer's characteristic that the virus could go across the neuronal synapses (7, 16-20), and these nuclei may be connected to the hippocampus over one or more synapses through the three major cholinergic nuclei projecting to the hippocampus. And these cholinergic neurons could be an important clue that the memory-related cholinergic functions may be controlled by or with higher cholinergic neurons innervating them.

In the present work, the labelling patterns were revealed similar in three different area groups of the hippocampus. Such findings suggest that the hippocampus may be received same cholinergic extrinsic innervations from the brainstem to cerebrum. Our data based on the morphological network could not explain whether all the systems may work at the same time or work separately. Further electrophysiological studies may be needed to reveal this mechanism.

In conclusion, the present study with PRV-Ba as a transneuronal tracer provides a new information about the location and distribution of the central cholinergic nuclei innervating the dentate gyrus, CA1, and CA3 of the hippocampus.

REFERENCES

1. Coyle JT, Price DL, Delong MR. *Alzheimer's disease: a disorder of cortical cholinergic innervation. Science 1983; 219: 1184-90.*
2. Voykto ML. *Cognitive functions of the basal forebrain cholinergic system in monkeys: memory or attention? Behav Brain Res 1996; 75: 13-25.*
3. Dougherty KD, Turchin PI, Walsh TJ. *Septocingulate and septohippocampal cholinergic pathways: involvement in working/episodic memory. Brain Res 1998; 810: 59-71.*
4. Lamour Y, Dutar P, Jobert A. *Septo-hippocampal and other medial septum-diagonal band neurons: electrophysiological and pharmacological properties. Brain Res 1984; 309: 227-39.*
5. Mesulam MM, Mefson EJ, Wainer BH, Levy AI. *Central cholinergic pathways in the rat: an overview based on an alternate nomenclature (Ch1-Ch6). Neuroscience 1983; 10: 1185-201.*
6. Martin X, Dolivo M. *Neuronal and transneuronal tracing in the trigeminal system of the rat using herpes virus suis. Brain Res 1983; 555: 253-76.*
7. Kim ES, Li H, McCulloch PF, Morrison LA, Yoon KW, Xu XM. *Spatial temporal patterns of transneuronal labelling in CNS neurons after injection of pseudorabies virus into the sciatic nerve of adult rat. Brain Res 2000; 857: 41-55.*
8. Broussard DL, Li H, Altsculer SM. *Colocalization of GABAA and NMDA receptors within the dorsal motor nucleus of the vagus nerve (DMV) of the rat. Brain Res 1997; 763: 123-6.*
9. Billing I, Foris JM, Card JP, Yates BJ. *Transneuronal tracing of neural pathways controlling an abdominal muscle, rectus abdominis, in the ferret. Brain Res 1999; 820: 31-44.*
10. Houser CR, Crawford GD, Barber RP, Salvaterra PM, Vaughn JE. *Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. Brain Res 1983; 266: 97-119.*
11. Levey AI, Wainer BH. *Cross-species and intraspecies reactivities of monoclonal antibodies against choline acetyltransferase. Brain Res 1982; 234: 469-73.*
12. Dougherty KD, Turchin PI, Walsh TJ. *Septocingulate and septohippocampal cholinergic pathways: involvement in working/episodic memory. Brain Res 1998; 810: 59-71.*
13. Hortnagl H, Groll-Knapp E, Khanakah G, Sperk G, Bubna-Littitz H. *Perception of species-specific vocalizations in rats: role of the cholinergic septohippocampal pathway and aging. Int J Dev Neurosci 1998; 16: 715-27.*
14. Brito GN, Brito LS. *Septohippocampal system and the prelimbic sector of frontal cortex: a neuropsychological battery analysis in the rat. Behav Brain Res 1990; 36: 127-46.*
15. Eichenbaum H, Otto T, Cohen NJ. *The hippocampus—what does it do? Behav Neural Biol 1992; 57: 2-36.*
16. Card JP, Rinaman L, Lynn RB, Lee BH, Meade RP, Miselis RR, Enquist LW. *Pseudorabies virus infection of the rat central nervous system: ultrastructural characterization of*

viral replication, transport, and pathogenesis. J Neurosci 1993; 13: 2515-39.

17. Jansen AS, Van Nguyen X, Karpitskiy V, Mettenleiter TC, Loewy AD. *Central command neurons of the sympathetic nervous system: basis of the fight-or-flight response. Science 1995; 270: 644-6.*
18. Card JP, Levitt P, Enquist LW. *Different patterns of neuronal infection after intracerebral injection of two strains of pseudorabies virus. J Virol 1998; 72: 4434-41.*
19. Card JP. *Practical considerations for the use of pseudorabies virus in transneuronal studies of neural circuitry. Neurosci Biobehav Rev 1998; 22: 685-94.*
20. Chen S, Yang M, Miselis RR, Aston-Jones G. *Characterization of transsynaptic tracing with central application of pseudorabies virus. Brain Res 1999; 838: 171-83.*
21. Loewy AD. *Pseudorabies virus: a transneuronal tracer for neuroanatomical studies. In: Viral Vectors, edited by Anonymous. New York: Academic Press, 1995; 349-66.*