The role of the supramammillary area of the hypothalamus in cognitive functions
Hyun Soo Shim, Hyun-Jung Park, Mi-Sook Lee, Minsook Ye and Insop Shim
Department of Physiology, College of Medicine, Kyung Hee University, Seoul, Korea

ABSTRACT
The supramammillary area (SUM) of the hypothalamus has wide spread connection with numerous brain structures. It is known that the SUM can control the frequency of the hippocampal theta rhythm, which plays a role in the cognitive functions of the hippocampal formation. In order to examine the role of the specific cells of the SUM in learning and memory, selective cholinergic neurotoxic or excitotoxic lesioned rats of the SUM were tested for spatial memory on the Morris water maze (MWM) test. After the behavior tests, the expression of acetylcholinesterase (AChE) in the hippocampus was studied using the immunohistochemistry. In the MWM test, both lesion of the SUM with 192 IgG-saporin or ibotenic acid produced the impairment of spatial learning and memory. The expression of AChE immunoreactive neurons in the hippocampal CA3 region was decreased after injections of 192 IgG-saporin into the SUM. These findings suggest that cholinceptive cells of the SUM area may play a critical role in the process of learning and memory.

Introduction
The supramammillary area (SUM) of the hypothalamus is a small nucleus that overlies the mammillary bodies medially. The SUM has widespread connections with numerous brain structures, including the brain stem, the dorsal raphe, the midbrain, the hypothalamic area, the basal forebrain, the limbic area and the cingulate cortices). It is suggested that the SUM may serve as an ‘interface’ for relaying various types of nonspecific multimodal inputs to the hypothalamic and limbic structures, which are involved in the control of behavior (Pan and McNaughton 1997).

The SUM can control hippocampal plasticity and it has also been recently shown to contain cells that determine the frequency of hippocampal rhythmical slow activity (theta rhythm) (McNaughton et al. 1995; Kirk 1998). Theta activity is likely to play a role in the cognitive and/or emotional functions of the hippocampal formation (Kirk and McNaughton 1991; Kocsis and Vertes 1994). Pan and McNaughton (1997) have reported that inactivation of the SUM produced a decrease in the theta frequency in freely moving rats and disruption of spatial learning. Also, electrolytic lesions of the SUM and adjacent nuclei or local injections of lidocaine to the SUM impaired learning and memory on a radial arm maze and a Morris water maze, and they impaired passive avoidance learning, which is all similar to hippocampal lesions (Jarrard 1983; Sziklas and Petrides 1993; Sziklas et al. 1996; Shahidi et al. 2004; Aranda et al. 2006). These studies suggest that the SUM may be critical for the cognitive aspects of hippocampal function.

The cholinergic system is known to be involved in the information processing related to hippocampal learning and memory (Oh et al. 2007). Learning and memory in mammals, including humans, is dependent on the neurotransmitter acetylcholine (ACh) in the basal forebrain and hippocampus (Donahue et al. 2004). It has been shown that cholinergic cells in the SuM region play a role in the baroreflex and potentiate the reflex rise in blood pressure to occlusion of the carotid artery, the so-called carotid arterial occlusion reflex (Ruggiero et al. 1990). Therefore, the biosynthetic enzyme, choline acetyltransferase (ChAT) or hydrolysing enzyme, acetylcholinesterase (AChE) for Ach, is currently the most reliable biomarker for cholinergic neuron activity (Wahba and Soliman 1992). The SuM region is involved in the control of hippocampal theta rhythms and modulates the synaptic excitation of hippocampal neurons (Mizumori et al. 1989; Kocsis and Vertes 1994; Jiang and Khanna 2006). Theta is a rhythmic, electrical field activity of the hippocampus with a well-established behavioral correlation (Borhegyi et al. 1997). The AChE or ChAT positive cells from the SuM region may modulate hippocampus-dependent behaviors. Both cholinergic-muscarinic and nicotinic receptors are localized in the SUM (Rotter et al. 1979; Dineley-Miller and Patrick 1990).
It has been demonstrated that injections of cholinergic agonist carbachol into the SUM elicited hippocampal theta rhythm and suppression of CA1 populations spike (Ikemoto et al. 2006; Ariffin et al. 2010). Positive reinforcement is mediated by the SUM nicotinic neurons (Ikemoto et al. 2006). However, roles of the cholinceptive neurons in the SUM in mediating process of learning and memory are not yet known.

The aim of the present study was to examine the role of the specific cells of the SUM in modulating learning and memory. Two types of manipulations by ibotenic acid which destroys non-selective cells or 192 IgG saporin destroying selectively cholinceptive neurons were utilized. Selective cholinergic neurotoxic or excitotoxic lesioned rats of the SUM were tested for spatial memory on the Morris water maze test (MWM). After the behavioral tests, the expression of AChE, a well-known target and biomarker for memory dysfunction or dementia (Shim et al. 2003; Kim et al. 2011) was studied using the immunohistochemistry.

Materials & methods

Animals

All the experimental procedures performed on the animals were conducted with the approval of the Ethics Committee of the Kyung Hee University and in accordance with the US National Institutes of Health ‘Guide for the Care and Use of Laboratory Animals’ (NIH Publication no. 80–23, revised 1996).

Subjects were male Sprague-Dawley rats (Orient Animal Corp, Kyunggido, Korea) that weighed 220–240 g each were used for the experiments. Rats were group-housed (three per cage) under a reversed light-dark cycle (light on from 08:00 to 20:00 h). The room temperature was 20°C–25°C and the humidity was 30% ± 5%. The rats had free access to food and water. All the rats were handled daily for at least a week prior to the experiment.

Surgery

A monoclonal antibody to the low-affinity nerve growth factor (NGF) receptor, 192-IgG, coupled to a saporin (192-IgG saporin, Millipore, U.S.A.), have been described as an efficient and selective immunotoxin for the NGF-receptor bearing cholinergic neurons in rat basal forebrain(Wiley et al. 1991). Ibotenic acid (Sigma, U.S.A.) is a neurotoxin which is used to destroy cell bodies. Ibotenic acid stimulates neuronal necrosis by a hyperstimulation of the NMDA glutamate receptors leading to calcium overload.

The skull was firmly placed in the apparatus, and the scalp was shaved and cleaned with betadine; an incision was made through the skin and muscle to expose the skull and the skin was then retracted. Ibotenic acid (IBO group, n = 10), 192 IgG-saporin (SAP group, n = 12) or its CSF vehicle (Control group, n = 5) in CSF (1 μl total volume) was microinjected via a 22-gauge Hamilton syringe (Reno, NV, U.S.A) at a rate of 0.1 μl/min into the SUM at the following coordinates: AP −4.8 mm, ML ± 0.6 mm, DV −8.7 mm relative to the bregma. All the employed coordinates were from the atlas of Paxinos and Watson (Paxinos and Watson 1986). After infusion of the neurotoxins, the needle was left in place for 10 min before it was slowly retracted. The remaining group was treated similarly, except that CSF was microinjected into the SUM. All animals were allowed to recover and the lesions were allowed to develop for 14 days following the surgery and prior to carrying out the experiments.

Morris water maze test

All the animals started training on the MWM task in a swimming pool (1.8 m diameter and 0.5 m high, filled with milky water at a temperature in the 22°C ± 2°C range) for 5 days. A 12 cm diameter round platform was hidden in a constant location (the quadrant center) within the pool with its top surface submerged 1.5 cm below the water level. The rats were trained to locate the hidden island during three trials per day for 4 days. After the 4 days, they were started in the quadrant opposite to the target and were forced to swim for 60 s in the pool without a platform. The spatial memory of the rats was assessed as the latency time and swimming distance on the fifth day. The time spent in the training quadrant, i.e. the previous location of the platform, was recorded and used as a measure of memory retention. A video camera was mounted on the ceiling above the pool and it was connected to a video-recorder and tracking device (S-MART; Pan-Lab, Barcelona, Spain), which permitted on-line and off-line automated tracking of the path taken by the animal (Chen et al. 2000).

Perfusion and immunohistochemistry

After behavioral testing was completed, all animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and then perfused through the ascending aorta with normal saline (0.9%), followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, postfixed overnight and cryoprotected in 20% sucrose in 0.1 M phosphate buffer saline (PBS) at 4°C. The following day, the brains were rapidly frozen and cut with a cryostat into 30 μm coronal.
sections through the hippocampus. Cryostat sections through the hippocampus were processed for the immunohistochemistry of AChE using methods we have previously described in detail (Shim et al. 2003).

The tissue was developed using diaminobenzadine as the chromogen with nickel intensification. In control sections the primary antibody was omitted or replaced with nonimmune sheep serum. In neither case was any specific labeling apparent. For counting the cells, a micrometer grid (200 × 200 μm) was placed according to the atlas of Paxinos and Watson under a light microscope.

Additional sections were taken through the injection site and stained with cresyl violet (ICN Biomedicals, Aurora, USA) to assess the degree of damage to the SUM and surrounding areas. Sections were mounted on gelatin-coated microscope slides, allowed to dry for 10 min and stained with cresyl violet for 5 min. They were then dehydrated in increasing ethanol concentrations and finally cover slipped from xylene.

**Statistical methods**

The data were reported as the means ± S.E.M. of the individual values of the rats from each group. SPSS 18.0 for Windows was used for statistical analysis. The significance level was set at *P* < .05. The behavioral data were statistically analyzed by one-way or multiple ANOVAs followed by LSD post hoc test. One-way ANOVA was used for the AChE-immunohistochemistry, followed by the LSD test.

**Results**

Histological data revealed that cannula tracts terminated within the SUM. An example of a typical placement is

![Figure 1](image)

**Figure 1.** (A) Schematic drawings of the region of the supramammillary complex by ibotenic acid and (B) cresyl violet stained photomicrographs showing the sites of neurotoxins injection in the SUM area 3V, 3rd ventricle; PR, prerubal filed; fr, fasciculus retroflexus; pm, principal mammillary tract; SUM, supramammillary; MM, medial mammillary nucleus; LM, lateral mammillary nucleus. Scale bar; 200 μm.
shown in Figure 1(A,B). The IBO group exhibited lesions mainly centered on the medial nucleus (80% of the extent of the nucleus), but substantial lesions were also observed in both lateral mammillary nuclei.

In order to demonstrate the role of specific cell types of SUM in mediating learning and memory, two types of manipulations by ibotenate, non-selective neurotoxin which destroys the most of cells and IgG-saporin which destroys selectively cholinergic neurons were utilized and AChE immunohistochemistry in the hippocampus was carried out.

The results of the Morris water maze task are depicted in Figure 2. As shown in Figure 2(A), the escape route of the rat in the control group was short and direct, while in the SUM-lesioned groups, the route was complex and wandering. In acquisition test, the escape latency and the escape distance of SUM-lesioned groups by ibotenic acid or 192 IgG-saporin were significantly longer than in the control group (Figure 2(B,C)). As shown in Figure 2(B), the escape latency differed among the groups averaged over all the sessions ($F_{2,26} = 12.1, P < .001$) and effect of day ($F_{3,78} = 3.4, P < .05$) but not group x day interaction. Post hoc comparisons revealed that the IBO group spent more time to reach the hidden platform in the MWM, compared with the control group ($P < .05$ on 2nd day and $P < .01$ on 4th day, respectively). Also, the SAP group spent more time to reach the platform in the MWM (2nd day and 4th day, $P < .05$, respectively). In Figure 2(C), the escape distance differed among the groups averaged over all the sessions ($F_{2,26} = 8.8, P < .001$), effect of day ($F_{3,78} = 2.9, P < .05$) but not group x day interaction. In the retention test, the spent time and total swimming distance of lesioned groups were significantly shorter than those in the control group ($F_{2,24} = 3.6, P < .05$ and $F_{2,24} = 6.0, P < .01$, respectively; Figure 2(B,C)).

The results of AChE immunoreactivity in the SUM-lesioned rats are depicted in Figure 3(A,B). The AChE immunoreactivity in the hippocampal CA1 was not different among groups. However, the AChE reactivity in the hippocampal CA3 was significantly different among groups ($F_{2,24} = 8.4, P < .05$). The expression of AChE in the hippocampal CA3 was significantly decreased in the SAP group compared to the control group ($P < .05$).

**Discussion**

The present study demonstrated that the lesion of the SUM with ibotenic acid or 192-IgG saporin produced the impairment of spatial learning and memory. Also, the expression of AChE in hippocampal CA3 was significantly decreased in the SAP group compared to the control. Our findings suggest that learning and memory deficit produced by injection of 192-IgG saporin may be closely associated with blockade of cholinergic transmission of the SUM.

The SUM connects with the septo-hippocampal system and it is involved in the control of the frequency of the hippocampal theta rhythm which plays a specific role in the cognitive functions of the hippocampal formation (Vertes 1992; Vertes and McKenna 2000). This suggests that the SUM can be regulated the function of the hippocampus through the direct connection and control of the hippocampal theta rhythm. McNaughton and Morris reported that large lesions at the core of the SUM decrease the theta frequency only modestly and they produce an effect on the spatial reference memory on the MWM (McNaughton and Morris 1987).

The electrical lesion of the SUM and the mammillary bodies impairs radial arm maze learning, which is similar to hippocampal lesions (Aranda et al. 2006). Also, temporary inactivation of the SUM using tetrodotoxin impairs the spatial working and reference memory retrieval in a delayed-matching-to-position task (Aranda et al. 2008). The lateral regions of SUM area were selected for lesion in the present study, since many studies have shown that the cells in lateral nucleus rather than the medial SUM are more important in mediating effect of SUM on hippocampal functions (Swanson 1982; Berger et al. 2001; Soussi et al. 2010). Consistent with other studies, the present study demonstrated that lesion of this region by neurotoxins severely altered learning and memory ability.

Ibotenic acid is a chemical compound that is naturally occurring in the mushrooms Amanita muscaria and Amanita pantherina, among others. It is a powerful neurotoxin that is used as a ‘brain-lesioning agent’ and has shown to be highly neurotoxic when injected directly into the brains of rodents. Ibotenic acid induces neuronal necrosis via hyperstimulation of the N-methyl-D-aspartate (NMDA) receptor leading to a calcium over-load. Its excitotoxic properties are confined to the somata of various neuron types. Therefore, the axons and blood vessels that course through the target area remain intact (Choi et al. 1988). In order to examine the role of the SUM in spatial learning and memory, ibotenic acid lesioned rats of the SUM area were tested for spatial memory on the Morris water maze (MWM) test. The IBO group showed poorer performance of acquisition and retention test than did the control group. Ibotenic acid-induced lesion of the SUM blocks to project from the SUM area to the hippocampus or indirectly to septo-hippocampal network, suggesting that the SUM plays a role in spatial learning and memory.

A monoclonal antibody to the low-affinity nerve growth factor (NGF) receptor, 192-IgG, coupled to a
saporin (192-IgG saporin), have been described as an efficient and selective immunotoxin for the NGF-receptor bearing cholinergic neurons in rat basal forebrain (Wiley et al. 1991). The present study demonstrated that cholinceptive cells in the SUM were involved for spatial learning and memory. In the current study, injection of

Figure 2. Results of the Morris water maze test. (A) Typical escape route (the swimming trajectory plot) in the each groups (B) Changes of the escape latency to reach hidden platform of the acquisition test and time spent in the target quadrant (C) Alterations of the escape distance to find the platform in the acquisition test and the total distance in the target quadrant. Three trials per day over 4 days were performed for the acquisition test. The task was performed with three daily trials on the 5th day without the platform for the retention test. Control group (n = 5) was microinjected with CSF; IBO group (n = 10) was received microinjections of ibotenic acid at the SUM; SAP group (n = 12) was received microinjections of IgG-saporin at the SUM. Significance of the acquisition test with LSD test following a repeated measures of ANOVA and significance of the retention test with LSD test following a one-way ANOVA are indicated as mean ± SEM. #P < 0.05 and ## P < 0.01, compared to the Control group.
192 IgG-saporin, selective cholinergic neurotoxin, into the SUM in rats affected the spatial learning and memory of rats in the MWM. The latencies to find the platform on acquisition trials by the SAP group were significantly increased compared to those of the control group. The SUM-lesioned group with 192 IgG-saporin also spent a lower proportion of the probe trial searching in the training quadrant.

192 IgG-saporin destroys selectively only cholinergic neurons, whereas ibotenic acid is non-selective neurotoxin which destroys the most of cells including GABAergic, glutamatergic or cholinergic cells. Therefore, it is possible that ibotenic acid-lesioned nonspecific cells including dopamine, 5-HT or glutamate and it cannot be excluded the possibility that these non-cholinergic cells may play also an important role in mediating learning and memory functions as seen in IBO group, and this suggestion was strengthen by the fact that serotonergic neurons in the SuM play an important role for spatial memory (Gutierrez-Guzman et al. 2017).

In the hippocampus, the expression of AChE immunoreactive cells was significantly reduced after injection of 192 saporin, and these results suggest that SUM directly affects to CA3 cells. The cholinergic systems in the forebrain are known to be involved in the formation and maintenance of short-term working memory as well as in the retention and retrieval processes of long-term reference memory. Our result demonstrated that 192 IgG-saporin induced selective lesion of cholinceptive cells in the SUM. And spatial memory impairment may be closely associated with cholinergic SUM injury. Therefore it can be concluded that cholinergic transmission in the SUM may play a critical role in spatial memory and synaptic plasticity. Supporting this conclusion, several functional studies have reported that SUM modulates CA1 pyramidal cells of the hippocampus (Jiang and Khanna 2006; Soussi et al. 2010). For example, cholinergic activation of SUM region suppressed CA1 cell excitability. The findings of the present study indicate that the effect is site-specific since it is confirmed that neurotoxins did not diffuse into the medial septum (MS) or other forebrain areas and the cells of the MS were intact after injection of 192 IgG-saporin or ibotenic acid (data were not shown).

It should be noted that the SUM cells that project to DG and CA2/CA3 also send collaterals to the MS (Vertes and McKenna 2000). The lateral SUM is the major source of afferents to the MS which then sends heavy projection to the hippocampus (Vertes 1992). It is possible that indirect pathway via the MS may also affect memory impairment and AChE decrease in the CA3 since several studies have shown that the MS modulates effects of SUM cells on synaptic excitability of hippocampus through the MS (Jiang and Khanna 2006).

In conclusion, degeneration of the cholinergic neurons in the forebrain is a common feature of Alzheimer’s disease and vascular dementia. Several animal model studies have attempted to clarify this cognitive

![Figure 3](image_url)

Figure 3. (A) The density of acetylcholinesterase (AChE) immunoreactive cells in the hippocampus. Control group (n = 5) was naïve; IBO group (n = 10) received microinjections of ibotenic acid at the SUM; SAP group (n = 12) received microinjections of IgG-saporin at the SUM. Significance with LSD test following a one-way ANOVA is indicated as mean ± SEM. #P < 0.05 compared to the Control group, (B) Photographs showing the distribution of AChE-immunoreactive cells in the hippocampus of Control group (A,D), IBO group (B,E), SAP group (C,F). Sections were cut coronally at 30 μm and the scale bar represents 200 μm.
impairment using basal forebrain lesions by192 IgG-saporin. In the present study, we demonstrated that the lesion of SUM region, one of specific major inputs of the hippocampus or septohippocampal network, induces the learning and memory deficits and reduced cholinergic neurotransmission from SUM cells has been correlated with cognitive decline. Therefore, our results may elucidate the role of cholinergic transmission in the SUM in mediating encoding of new memories and synaptic plasticity.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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