Effects of long‑term scopolamine treatment on cognitive deficits and calcium binding proteins immunoreactivities in the mouse hippocampus

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Abstract. GABAergic projections terminate on numerous hippocampal interneurons containing calcium binding proteins (CBPs), including calbindin D-28k (CB), calretinin (CR) and parvalbumin (PV). Memory deficits and expression levels of CB, CR, and PV were examined in the hippocampal subregions following systemic scopolamine (Scop; 1 mg/kg) treatment for 4 weeks in mice. Scop treatment induced significant memory deficits from 1 week after Scop treatment. CB, CR, and PV immunoreactivities distributions were in hippocampal subregions [CA1 and CA3 regions, and the dentate gyrus (DG)]. CB immunoreactivity (CB+) was gradually decreased in all subregions until 2 weeks after Scop treatment, and CB+ was decreased to the lowest level in all subregions at 3 and 4 weeks. CR+ in the CA1 region was gradually decreased until 2 weeks and hardly observed at 3 and 4 weeks; in the CA3 region, CR+ was not altered in all subregions at any time. In the DG, CR+ was gradually decreased until 2 weeks and lowest at 3 and 4 weeks. In the CA1 region, PV+ did not change in any subregions at any time. In the DG, PV+ was not altered at 1 week, and gradually decreased from 2 weeks. In the CA3 region, PV+ did not change in any subregions at any time. In brief, Scop significantly decreased CBPs expressions in the hippocampus ≥3 weeks after the treatment although memory deficits had developed at 1 week. Therefore, it is suggested that Scop (1 mg/kg) must be systemically treated for ≥3 weeks to investigate changes in expression levels of CBPs in the hippocampus.

Introduction

Muscarinic acetylcholine receptors (mAChRs) are primary receptors in cholinergic transmission, and mAChRs immunoreactivities in the hippocampus are present in pyramidal cells, granule cells, and GABAergic interneurons (1). It is well established that systemic injection of scopolamine (Scop), a muscarinic cholinergic receptor antagonist, produces learning and memory deficits through dysregulation of cholinergic mechanisms in the hippocampus (2).

Septo-hippocampal cholinergic pathway, which originates in the medial septum/diagonal band, is one of crucial circuits for cognitive function (3). Also, it is well known that septo-hippocampal GABAergic pathway plays important roles in learning and memory processes (4). Important inputs to the hippocampus are provided by GABAergic fibers as
well as cholinergic fibers from the medial septum, and most of GABAergic projections terminate on many hippocampal interneurons including neurons containing calcium binding proteins (CBPs), such as calbindin D-28k (CB), calretinin (CR), and parvalbumin (PV) (5-7). GABAergic nonpyramidal cells transmit hippocampal feedback to GABAergic neurons in the medial septum, which forms septo-hippocampo-septal circuit that regulates the activity of GABAergic septo-hippocampal projections (8).

The maintenance of intracellular calcium metabolism in the hippocampus is an important factor in synaptic plasticity and memory performance (9,10). Alterations in numbers or expressions of CBPs in hippocampal neurons are observed in post-mortem brain tissues in schizophrenia (11) and kainate-induced seizures (12), and these changes contribute to cognitive impairments in neurological disorders (13,14). However, in Scop-induced amnesia models, alterations in CBPs expressions have not been fully studied.

In the present study, therefore, we examined temporal and spatial alterations of CBPs expressions in hippocampal subregions after chronic systemic treatment with Scop in mice, and investigated the relationship between hippocampal CBPs expressions and memory deficits after Scop treatment.

**Materials and methods**

**Experimental animals.** Male ICR mice (8-week-old) were purchased from Orient Bio Inc. (Seongnam, South Korea) and used after 7 days of acclimation. The procedures for animal handling and care adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th edition, 2011), and the experimental protocol of the present study was reviewed and approved based on ethical procedures and scientific care by the Kangwon National University-Institutional Animal Care and Use Committee (approval no. KW-160802-3).

**Experimental groups and drug treatment.** Experimental mice were divided into five groups (n=14 at each point in time per group): i) Control group; ii) 1 week (wk)-Scop group, which received Scop treatment for 1 week; iii) 2 wk-Scop group; iv) 3 wk-Scop group; and v) 4 wk-Scop group. Normal saline or 1 mg/kg Scop was dissolved in normal saline and intraperitoneally injected once a day for 1 to 4 weeks (15-17). The mice in each group were sacrificed 24 h after the final administration.

**Behavioral performance**

**Morris water maze test (MWMT).** The MWMT for spatial memory was performed according to our published procedure (18). The animals (n=7) in each group were tested 1 day before sacrifice following training for 1 day. Briefly, we used the Gemini Avoidance system (GEM 392; San Diego Instruments, San Diego, CA, USA) that consists of light and dark compartments with a grid floor. For training, the animals in each group were allowed to explore environments in both light and dark compartments for 1 min and given an inescapable foot-shock (0.3 mA for 3 sec) after entering the dark compartment. The animals were tested 15 min after the training without applying the foot-shock. The interval between the beginning of the test and the entry into the dark compartment was defined as the latency time.

**Immunohistochemistry.** Immunohistochemistry for CB, CR, and PV was carried out according to our published method (20). Briefly, mice (n=7 in each group) were anesthetized with 30 mg/kg Zoletil 50 (Virbac, Carros, France) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Their brains were serially sectioned into 30-μm coronal sections in a cryostat (Leica Microsystems GmbH, Wetzlar, Germany). The sections were incubated with diluted goat anti-CB (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-CR antibody (1:1,000; Chemicon International, Inc., Temecula, CA, USA) or goat anti-PV (1:200; Santa Cruz Biotechnology) overnight and subsequently exposed to biotinylated rabbit anti-goat or goat anti-rabbit IgG, and streptavidin peroxidase complex (1:200; both from Vector Laboratories, Inc., Burlingame, CA, USA). In order to establish the specificity of the immunostaining, a negative control resulted in the absence of immunoreactivity in the stained sections.

**Data analysis.** As previously described (19), digital images of CB-, CR-, and PV-immunoreactive structures were captured with an AxioM1 light microscope equipped with a digital camera (Axiocam; both from Carl Zeiss AG, Oberkochen, Germany) connected to a PC monitor. The density of each immunoreactive structures was evaluated on the basis of optical density (OD), which was obtained after the transformation of the mean gray level using the formula: OD=log (256/mean gray level). After the background was subtracted, a ratio of the OD of image file was calibrated as % [relative optical density (ROD)] using Adobe Photoshop version 8.0 and NIH Image J software (National Institutes of Health, Bethesda, MD, USA). The mean value of the OD of the control group was designated as 100%, and the ROD of each group was calibrated and expressed as % of the control group.

**Statistical analysis.** The data shown here represent the means ± SEM. Differences of the means among the groups were statistically analyzed by analysis of variance with a post hoc Bonferroni’s multiple comparison test in order to elucidate effects of long-term Scop treatment among groups. Statistical significance was considered at P<0.05.

**Results**

**MWMT.** Spatial memory was evaluated using the MWMT. Escape latency in all the Scop treated groups was significantly increased compared to that in the control group (Fig. 1Aa and Ab). In addition, duration of the time in the target quadrant was significantly decreased in all the Scop treated...
groups (Fig. 1Aa and Ac). However, there were no significant differences among the Scop treated groups (Fig. 1Ab and Ac).

**PAT.** Short-term memory was evaluated using PAT. Similar to results of the MWMT, latency in all the Scop treated groups was significantly decreased compared to that in the control group (Fig. 1B), however, the latency was not significantly different among the Scop treated groups (Fig. 1B).

**CB immunoreactivity.** CB immunohistochemistry results across weeks 1 to 4 are presented in Fig. 2. In the control group, CB immunoreactivity was identified in neurons of the stratum pyramidale (SP) in the CA1 region, in mossy fibers of the stratum lucidum in the CA3 region, and in cells of the granule cell layer (GCL) in the dentate gyrus (DG) (Fig. 2Aa-Ac). In the 1 wk-Scop group, the distribution pattern of CB immunoreactivity in all the subregions was similar to that in control group, however, the CB immunoreactivity in all the subregions was significantly decreased compared to that in the control group (Fig. 2Ba-Bc and F). In the 2 wk-Scop group, CB immunoreactivity was more reduced compared with that in the 1 wk-Scop group (Fig. 2Ca-Cc and F). In the 3 wk- and 4 wk-Scop groups, CB immunoreactivity was hardly shown in the CA1 and CA3 regions (Fig. 2Da, Db, Ea, Eb and F), and very weak in the DG (Fig. 2Dc, Ec and F).

**CR immunoreactivity.** CR immunohistochemistry results across weeks 1 to 4 are presented in Fig. 3. In the control group, CR immunoreactivity in the CA1 and CA3 regions was shown in cells scattered in the strata oriens and radiatum (Fig. 3Aa and Ab), and, in the DG, CR immunoreactivity is found in the GCL and cells scatted in the polymorphic layer (PL) (Fig. 3Ac). In the Scop groups, CR immunoreactivity was gradually decreased with time in the CA1 region (Fig. 3Ba, Ca, Da, Ea and F), not significantly altered in the CA3 region (Fig. 3Bb, Cb, Db, Eb and F). In the DG, CR immunoreactivity was gradually decreased, in particular, CR immunoreactivity in the 3- and 4-wk Scop groups was shown in a few cells in the PL (Fig. 3Bc, Cc, Dc, Ec and F).

**PV immunoreactivity.** PV immunohistochemistry results across weeks 1 to 4 are presented in Fig. 4. In the control group, strong PV immunoreactivity was shown in cells scattered in the CA1 and 3 regions (Fig. 4Aa and Ab), and in the DG (4Ac). In addition, weak PV immunoreactivity was detected in the SP in the CA1 and 3 regions (Fig. 4Aa and Ab), and in the GCL in the DG (Fig. 4Ac). In the Scop groups, PV immunoreactivity in the CA1 region was similar to that in the control group at 1 week (Fig. 4Ba and F), thereafter, PV immunoreactivity in the CA1 region was gradually and significantly reduced with time (Fig. 4Ca, Da, Ea and F). In the CA3 region, PV

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Figure 1. Memory test using the MWMT (Aa‑Ac) and PAT (B) in the control and Scop groups. In the MMWT, escape latency in all the Scop groups is significantly increased compared with that in the control group (Aa and Ab), and duration of the time in the target quadrant in all the Scop groups is significantly decreased compared with that in the control group (Ac). In the PAT, latency in all the Scop groups is significantly decreased compared with that in the control group (B). (n=7 per group; *P<0.05 vs. the control group). The bars indicate the SEM. MWMT, Morris water maze test; PAT, passive avoidance test; Scop, scopolamine.
immunoreactivity was not significantly altered at any time after Scop treatment (Fig. 4Bb, Ch, Db, Eb and F). In the DG, PV immunoreactivity was not changed at 1 week after Scop treatment (Fig. 4Bc and F), and significantly reduced from 2 weeks after Scop treatment (Fig. 4Cc, Dc, Ec and F).

**Discussion**

In the present study, we investigated long-term changes of spatial and short-term memory, and CBPs expressions in the hippocampal subregions for 4 weeks after systemic Scop treatment in mice and found that significant spatial and short-term memory impairments were caused at 1 week post-treatment and maintained until 4 weeks post-treatment using the MWMT and PAT. These results were consistent with a previous study that showed that long-term Scop treatment induced spatial memory deficits at the beginning of 1 week after Scop injection (21). Acetylcholine has been implicated in memory, for instance, hippocampal-dependent memory (2). It has been well established that systemic Scop injection...
produces learning and memory impairments via dysregulation of cholinergic mechanisms in the hippocampus (2). For modulation of memory impairments, on the other hand, it has been reported that intraseptal muscarinic agonists alleviate systemic Scop-induced memory impairments (22). In addition, the septo-hippocampal GABAergic fibers are more excited by exogenous muscarine (4), and excitation of septo-hippocampal GABA neurons produces a powerful disinhibition of pyramidal neurons and promotes long-term potentiation in the hippocampus via hippocampal interneurons (4,22). Based on these reports, it is likely that modulation of muscarinic receptors could affect Scop-induced memory impairments through hippocampal interneurons. We, therefore, examined alterations of CB, CR, and PV immunoreactivities in the mouse hippocampus to investigate the link between cognitive functions and hippocampal interneurons containing CBPs after long-term systemic Scop treatment.

In the present study, CB immunoreactivity was significantly decreased in all hippocampal subregions from 1 week after Scop treatment, and rarely observed from 3 weeks after Scop...
treatment. We recently reported that muscarinic acetylcholine receptor M1 (M1R) was expressed in neurons of the SP and granular cell layer, the expression pattern was similar to that of CB in the CA1 area and DG, and Scop treatment significantly reduced M1R immunoreactivity in the hippocampus (23). On the other hand, a previous study reported that decreased CB immunoreactivity is gradually and significantly decreased in the CA1 region and the DG, not in the CA3 region, from 2 weeks after Scop treatment. Scop, scopolamine; DG, dentate gyrus; GCL, granule cell layer; ML, molecular layer; PL, polymorphic layer; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; ROD, relative optical density.

We found, in this study, that CR and PV immunoreactivities were also severely decreased in the CA1 region and in the DG from at least 3 weeks after Scop treatment; in the CA3 region, the immunoreactivities were not significantly altered until 4 weeks after Scop treatment. Pascual et al reported that CR- and PV-immunoreactive interneurons located in the CA1-3 region selectively received GABAergic axons originated from the medial septum in mice (5). In addition, it was reported...
that CBPs containing interneurons in the hippocampus were involved in inhibition of GABAergic septo-hippocampal neurons, which were the source of disinhibition of pyramidal neurons in the hippocampus in the mouse, rat and monkey (5-7). Furthermore, some researchers reported that about 50% of all muscarinic cholinocceptive nonpyramidal neurons contained PV, and about 90% of the PV containing neurons expressed mACHR in rats (25,26). Those previous findings as well as our present results suggest that markedly decreased CR and PV immunoreactivities in the CA1 region and in the DG of the mouse might be related to Scop-induced memory impairments. However, it needs to study what maintained CR and PV immunoreactivity in the CA3 region during chronic Scop treatment is related to in memory after or during Scop treatment.

Finally, in the present study, we found that PV immunoreactivity was not changed in all hippocampal subregions 1 week after Scop treatment, not like CB and CR immunoreactivities that were decreased from 2 weeks after Scop treatment. To the best of our knowledge, it is likely that PV containing neurons are relatively resistant to short-term (1 week) Scop treatment than CB and CR containing neurons in the hippocampus.

In conclusion, this study identified that CBPs immunoreactivities in the mouse hippocampus were differently decreased during period of systemic Scop treatment, and suggests that Scop-induced CBPs changes or reductions might be differently related according to types of CBPs to spatial and short-term memory deficits.

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