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RESEARCH ARTICLE

Modulation of spatial memory and expression of hippocampal neurotransmitter receptors by selective lesion of medial septal cholinergic and GABAergic neurons

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Abstract

The medial septum (MS) is an important modulator of hippocampal function. The degree of damage in which the particular set of septo-hippocampal projections contributes to the deficits of spatial memory with concomitant changes of hippocampal receptors expression has not been studied till present. Therefore, we investigated spatial memory and the expression level of cholinergic (α7 nACh and M1), GABAergic (α1 subunit of GABAA) and glutamatergic (NR2B subunit of NMDA and GluR 1 subunit of AMPA) receptors in the hippocampus following selective lesions of cholinergic and GABAergic septo-hippocampal projection. Learning process and long-term spatial memory were assessed using a Morris water maze. The obtained results revealed that in contrast to cholinergic lesions, rats with MS GABAergic lesions exhibit a retention deficit in 3 days after training. Western blot analyses revealed the MS cholinergic lesions have significant effect on the expression level of the M1 mACh receptors, while MS GABAergic lesions induce dramatic modulations of hippocampal glutamatergic, cholinergic and GABAergic receptors expression. These results for the first time demonstrated that selective lesions of MS cholinergic and GABAergic neurons differentially affect long-term spatial memory and the memory deficit after MS GABAergic lesion is paralleled with significant changes of hippocampal glutamate, GABA and acetylcholine receptors expression.

Keywords Spatial memory · Medial septum · Hippocampal receptors · Rat

Introduction

The role of hippocampus in various aspects of cognitive function, including learning and spatial memory, has been pointed out by extensive clinical and experimental data (Morris et al. 1982; Broadbent et al. 2004; Morgado-Bernal 2011). The various neurotransmitter systems within the hippocampal formation—glutamate, gamma-aminobutyric acid (GABA) and acetylcholine (ACh)—are of special importance in learning process and memory formation (Tamminga et al. 2012; Kim et al. 2012; Luscher and Malenka 2012; Malikowska-Racia et al. 2018).

The function of hippocampus is determined by its internal neural network as well as by its afferent and efferent connections. The medial septum (MS) is an important modulator of hippocampal function: the hippocampus receives fibers from the MS via the fimbria–fornix (Hangya et al. 2009; Roland and Savage 2009; Kanju et al. 2012). Septo-hippocampal (SH) projections are predominantly cholinergic and GABAergic (Rye et al. 1984), although there are some glutamatergic

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(Sotty et al. 2003) and neuropeptide projection neurons as well (Peterson and Shurlow 1992). Lesions or inactivation of MS neurons impair hippocampal-dependent forms of learning and memory (Lecourtier et al. 2011).

The main sources of acetylcholine release in the hippocampus are septal cholinergic neurons (Sun et al. 2014). The activation of SH cholinergic system can activate both metabotropic muscarinic (mAChRs) and ionotropic nicotinic (nAChRs) acetylcholine receptors in the hippocampus (Frotscher et al. 2000; Araque et al. 2002; Bell et al. 2011; Gu and Yakel 2011). The α7 nAChR subtype is prominent in the hippocampus, and its altered function has been linked with cognitive deficits and a variety of neurological disorders and diseases (Freedman and Goldowitz 2010; Sadigh-Eteghad et al. 2015). M1 muscarinic receptors are also involved in hippocampus memory processes (Nathan et al. 2013).

As mentioned above, the hippocampus, in addition to cholinergic fibers, receives GABAergic fibers from the MS. GABAergic SH projection targets only hippocampal interneurons in all hippocampal subfields (Freund and Antal 1988). Hippocampal GABAA receptor complexes play an essential role in spatial learning and memory, in which targeted disruption of the α1 subunit produces profound deficits (Ghafari et al. 2017).

In spite of the long history of septal research in memory function, the alteration of hippocampus-dependent spatial memory following selective lesions of different subsets of SH projections and concomitant changes of hippocampal receptor expression has not been studied. To address this question, in the present experiments we have employed two types of selective lesions, namely selective lesions of cholinergic or GABAergic SH projections and studied how they may differently affect spatial learning and memory and the expression of the following neurotransmitter receptors: cholinergic (α7 nACh and M1), GABAergic (α1 subunit of GABAA) and glutamatergic (NR2B subunit of NMDA and GluR 1 subunit of AMPA) receptors in the hippocampus of behaviorally characterized rats. As far as we know, no such a complex study has been performed before and the obtained data could lead to better understanding of the mechanisms by which SH projections can modulate memory.

Materials and methods

Animals

The animals were procured from the Laboratory Animal Division of I. Beritashvili Center of Experimental Biomedicine. A total of 36 male outbred albino rats, approximately 4 months of age and weighing 220–250 g at the start of experimentation, served as subjects. All experimental procedures were conducted in accordance with the European Communities Council Directive Guidelines for the care and use of laboratory animals (2010/63/EU—European Commission) and approved by the animal care and use committee at the I. Beritashvili Center of Experimental Biomedicine. The rats were kept in a polyacrylic cage (22.5 × 37.5 cm) in groups of four rats per cage and maintained under standard housing conditions (room temperature 22–25 °C and humidity 60–65%) with a 12 h light and dark cycle. Food and water were available ad libitum. Rats were randomly assigned to either the control or MS lesioned groups. There were 12 rats in each of three independent groups: (1) sham operated—control (Contr), (2) 192 IgG-saporin MS lesioned (SAP) and (3) GAT1-SAP MS lesioned (GAT). At the end of the behavioral experiments, half of the rats from each group were used in the immunohistochemical ($n = 6$) and half in immunoblotting ($n = 6$) studies. Schematic representation of the experimental design is shown in Supplementary Materials-1: Fig. S1.

The number of animals for behavioral, immunohistochemical and biochemical studies was estimated on our previous analog studies (see Dashniani et al. 2015, 2020) and is minimal for adequate statistical analysis.

Surgery

Rats were anesthetized with intraperitoneal injection of ketamine–xylazine (80 mg/kg and 10 mg/kg, respectively) and placed in a stereotaxic apparatus with the rat adaptor and lateral bars. All injections for immunolesion surgeries were performed stereotaxically according to Paxinos and Watson (1998) stereotaxic atlas and were corrected in our pilot experiments. Injection of GAT1-SAP (325 ng/µl, 0.5 µl; 0.05 µl/min) for selective immunolesions of GABAergic neurons or mouse saporin (this product serves as a control for the immunotoxin) for control surgeries (Advanced Targeting System, San Diego, USA) was performed from the side by a 15 degree angle (Pang et al. 2011) with the following coordinates AP—0.4; ML—1.7; DV—6.4. Injection of 192 IgG-saporin (1 mg/ml in phosphate-buffered saline) to produce selective lesion of MS cholinergic neurons was performed bilaterally at two depths: AP—0.45; ML—0.25; DV—7.8 (0.3 µl; 0.05 µl/min) and DV—6.2 (0.2 µl; 0.05 µl/min). After injection, the needle was left in place for an additional 8 and 5 min, respectively, to allow the toxin to diffuse from the injection site. All injections were made with a 1-µl Hamilton syringe with a microinjection pump (CMA 402 Syringe Pump, Sweden). For analgesia, the rat was given a 0.1 mg/kg injection of buprenorphine after the surgery. The rats were allowed to recover from the surgery for 10 days before starting the behavioral experiments.

Morris water maze

Long-term spatial memory was assessed by using a Morris water maze (Morris et al. 1982) which consists of a circular tank (1.5 m in diameter and 0.5 m height) filled with opaque (white-colored) water. Before behavioral experiments, each rat was gently handled by the experimenter a few minutes per day for 10 days in an experimental room to prevent neophobic reactions. The room, in which the tank was stationed, had sufficient number of cues (door, window, furniture, posters on the walls, etc.) to provide spatial cues. The task was adapted from Ge et al. (2010). Briefly, the test was divided into the training phase (days 1 and 2) and the retrieval phase. The escape platform (10 cm in diameter) was located 2 cm beneath the surface on the training day. On each test day, the animals received a block of four consecutive trials (1–4 trials—block 1; 5–8 trials—block 2), one from each of four equidistantly located start locations (N, S, E, W) in a randomized sequence (Fig. 1a). The rats were placed into the water facing the wall of the maze. The trial ended when the rat climbed on the available platform or until 60 s had elapsed. If a rat could not find the platform after 60 s, it was placed on the platform by the experimenter. Rats were left on the platform for 15 s and then were moved to a holding cage for a 2-min intertrial interval. The retention was tested in a probe trial 1 h or 3 days after task acquisition, during which the platform was removed from the pool. The rats were placed in the pool from a novel drop point (between the N and E compass points) and allowed to swim for 60 s. The time spent in each of the four quadrants was recorded and used for further analysis of memory retention performance. Tracking the animal movements in water maze, also collection of other numeric data (escape latency, the time spent in different quadrants during the probe test) was made with an aid of video tracking system designed and installed in our laboratory.

Immunohistochemistry

The 192 IgG-saporin or GAT1-SAP lesions of the MS were evaluated by decreased choline acetyltransferase (ChAT) or parvalbumin (PV) staining of the MS sections, respectively. In addition, staining for acetylcholinesterase (AChE) was performed in the hippocampus; hippocampal AChE was used as a quantitative measure of lesion extent.

The immunostaining was run in a random sample ($n=6$) for each group of animals. At the end of the behavioral experiments randomly assigned, half of rats from each group ($n=6$) were deeply anesthetized with pentobarbital and perfused through the ascending aorta with 300 ml saline followed by 600 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected out, postfixed at 4 °C for an additional 3–4 h in the same fixative, and stored frozen (− 70 °C) until usage. The brains were cut in coronal plan on a freezing microtome. The sections of the MS and hippocampus (20 µm thick) were placed in phosphate buffer for 30 min and mounted on polylysine-coated slides. All reagents and buffers were purchased from Santa

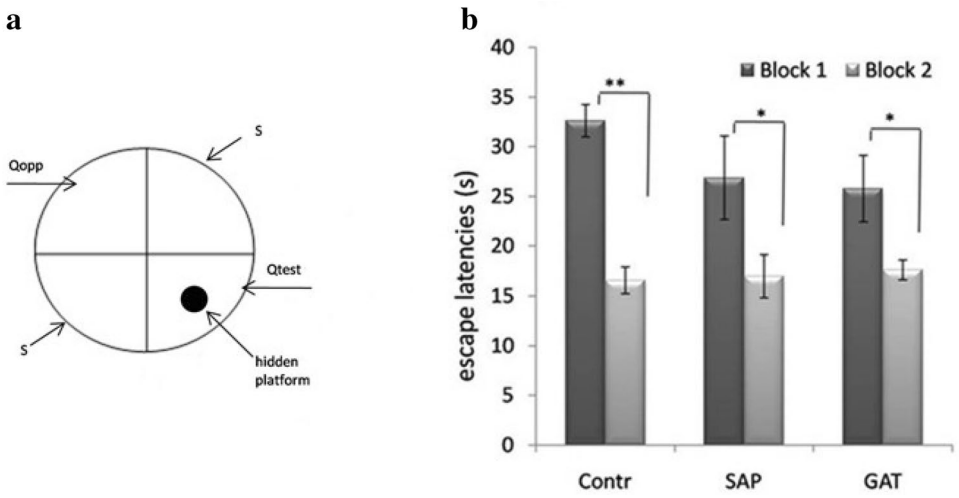

Fig. 1 **a** The selective lesions of cholinergic and (or) GABAergic SH projections did not prevent learning of a platform location in MWM. **a** The schematic shows the location of test and opposite quadrants (Q_{test}, Q_{opp}) and the location of the hidden platform. *s* the starting points for the probe test. **b** Histograms display the average escape latencies of rats for the first four (block 1) and last four (block 2) training trials. The decrease in the escape latency between block 1 (day 1: 4 trials) and block 2 (day 2: 4 trials) in MWM training task was taken to be a measure of the learning process. The results of paired *t* test analysis (after ANOVA) separately in three groups showed significant difference between block 1 and block 2 in the rats of control (***$p<0.001$), SAP (**$p<0.01$) and GAT (*$p<0.05$) groups

Cruz Biotechnology, Inc. (USA), and immunostaining was conducted with appropriate protocols (https://www.scbt.com/resources/protocols/immunoperoxidase-staining). In brief, coronal sections were treated in cold acetone, rinsed in phosphate buffer saline (PBS) and incubated in 5% normal blocking serum for 1 h. The brain sections were incubated in primary antibodies overnight at 4 °C. MS sections were incubated in primary antibodies to ChAT (1:500 dilution, sc-20672) and to PV (1:500 dilution, sc-7448) and a set of coronal hippocampal sections was processed using a primary antibody against AChE (1:600 dilution, sc-11409). The sections were then washed in PBS, incubated in appropriate secondary antibodies [for PV goat secondary antibody and ABC Staining System (sc-2023) and for ChAT and AChE rabbit secondary antibody and ABC reagent (sc-2018)] for 45 min. Standard immunocytochemical procedure was carried out with ABC reagent peroxidase substrate and chromogen mixture. Sections were then dehydrated through alcohols and xylenes, then one to two drops of permanent mounting medium was added and covered with a glass coverslip. Totally, six to ten sections of hippocampal and MS levels within experimental and control animals were selected and used to assess the effect of MS lesion on ChAT and PV-stained neurons in the MS and AChE-positive cells in the hippocampal CA1 and CA3 areas. The two-dimensional counting grid (250 µm × 250 µm) at the magnification 400 × was used. The sections were analyzed with a microscope Leica MM AF.

Electrophoresis and immunoblotting

The electrophoresis and immunoblotting were run in a random sample ($n = 6$) for each group of animals. The animal was deeply anesthetized via the respiratory route by exposure to ~ 15 ml diethyl ether for approximately 2 min with continuous monitoring of heart rate. Rat was decapitated when heart rate was reduced to approximately one beat per second. After decapitation, the hippocampus was removed and frozen immediately on dry ice. Tissue from hippocampus and single rat formed a single sample. P2 plasma membrane–mitochondrial fractions were obtained as described in Meparishvili et al. (2015). P2 fractions were dissolved in 5% solution of sodium dodecyl sulfate (SDS). Samples were then coded and all subsequent procedures performed without knowledge of the rats' experimental history. In all fractions, protein concentration was determined in quadruplicate using a microbicinchoninic acid protein assay kit (Pierce). Aliquots containing 30 µg of protein and of equal volume were applied to the gels. SDS gel electrophoresis and Western blotting were carried out as described previously (Meparishvili et al. 2015). After protein had been transferred onto nitrocellulose membranes, the membranes were stained with Ponceau S solution and analyzed with Image J software (https://imagej.net/ImageJ) to confirm transfer and the uniform loading of the gels. The membranes were then washed with phosphate-buffered saline + 0.05% Tween 20 and cut according to the molecular weight standards in three parts to carry out separate immunostaining with the used antibodies. The upper part of the filters were stained with antibodies against NR2B (ab65783; Abcam), the second part with monoclonal antibody against GluR1 (ab31232; Abcam), and the third part with antibodies either against acetylcholine muscarinic receptor M1 (ab75178; Abcam), α1 subunit of GABAA receptors (ab33299; Abcam) or α7 subunit of nACh (ab23832; Abcam). As the molecular weights of acetylcholine muscarinic receptor M1, α1 subunit of GABAA receptors and α7 subunit of nACh are near each other, separate electrophoresis and blotting were carried out for their study. Standard immunochemical procedures were carried out using peroxidase-labeled secondary antibodies and Super-Signal West Pico Chemiluminescent substrate (Pierce). The blots were then exposed with intensifying screens to X-ray films pre-flashed with Sensitize (Amersham). The optical densities of bands corresponding to the NR2B, GluR1, M1, α1 GABAA, and α7 nACh were measured using LabWorks 4.0 (UVP). The autoradiographs were calibrated by including in each gel four standards comprising the P2 fraction from the brain of untreated rats. Each standard contained 15–60 µg total protein. Optical densities were proportional to the amount of studied receptors. To obtain the data given in Figs. 4, 5, 6, 7, 8 and 9, the optical density of each band from an experimental sample (e.g., hippocampus of control rats) was divided by the optical density, which, from the calibration of the same autoradiograph, corresponded to 30 µg of total protein in the standard. The data expressed in this way will be referred to as "relative amount" of NR2B, GluR1, M1, α1 GABAA, and α7 nACh.

Data from experimental stained bands were not normalized with respect to actin or any other housekeeping protein, because it cannot be guaranteed that such proteins are unaffected by our experimental conditions (see also: Dittmer and Dittmer 2006; Li and Shen 2013; Ghosh et al. 2014; Chen and Xu 2015 for discussion of the unreliability of normalization to housekeeping proteins). Instead, we controlled loading by Ponceau S staining and calibrated with protein standards (see also, Meparishvili et al. 2015).

Statistical analysis

Statistical analysis was performed using the SigmaStat statistical software. The effect of treatment on the learning process and on the probe-test performance indices 1 h or 3 days after training were analyzed by two-way analysis of variance (ANOVA) with treatment (groups: control, MS-SAP, MS-GAT) as between-subject factors and training day (block 1 and block 2), as well the differences for time spent

in the test (Q_{test}) and opposite (Q_{opp}) quadrants during the probe test as the within-subject factor. Further post hoc comparisons or paired *t* test were used, where appropriate. For each group, time spent in the test quadrant with chance level (15 s) at each probe test were compared using one-sample *t* test. Two-sample *t* test was used to compare immunohistochemical data between control and lesioned groups. The protein data were analyzed by one-way ANOVA, with the factor—treatment. In case of significant effect in ANOVA, planned comparisons were done by two-tailed *t* test. All data are presented as mean ± standard error of the mean (SEM). Differences were considered significant when $p < 0.05$.

Results

Histology

Analyses of the number of ChAT- and PV-positive neurons in the MS

To determine the selective toxicity of the GAT1-SAP and 192 IgG saporin, Chat and PV immunoreactive (ChAT-ir and PV-ir) neurons were counted in the MS (Supplementary Materials-1: Table S1; Fig. S2). GAT1-SAP significantly reduced the number of PV-ir MS neurons by 80% and the number of ChAT-ir neurons only by 22% as compared to the control group ($t = 16.982$, $p < 0.001$; $t = 2.834$, $p < 0.05$, respectively). On the contrary, intraseptal 192 IgG saporin reduced the number of ChAT-ir neurons by 91% and spared most of the PV-ir neurons representing GABAergic MS neurons. Correspondingly the reduction of ChAT-ir neurons in the MS vs. control group was significant ($t = 17.372$, $p < 0.001$), whereas the changes in PV-ir neurons was not significant. Thus, the toxins predominantly affected their respective target neurons.

Analyses of the AChE activity in the hippocampus

Injection of 192 IgG-saporin into the MS causes significant reduction of AChE-positive neurons in the hippocampus vs. control group (CA1: $t = 38.607$, $p < 0.001$; CA3: $t = 8.220$, $p < 0.001$). GAT1-SAP MS lesions cause no significant reduction of AChE-positive neurons in the CA3 field of the hippocampus ($t = 1.599$, $p = 0.171$), but induces significant reduction in the CA1 field vs. control group ($t = 3.638$, $p = 0.015$). The loss of AChE-sensitive neurons in the hippocampus is less than that after 192 IgG-saporin lesions: differences between these groups in AChE staining in the CA1 field of the hippocampus were significant ($t = 12.380$, $p < 0.001$; Supplementary Materials-1, Table S2, Fig. S3).

Behavioral study

Selective lesions of cholinergic and GABAergic MS neurons did not prevent learning of a platform location in MWM

Selective immunolesions of the MS neurons were used to examine the relative contributions of cholinergic and GABAergic MS neurons to spatial memory formation in a Morris water maze (MWM) task. We used a 2-day MWM training protocol (Ge et al. 2010) with eight training trials. The decrease in the escape latency between block 1 (day 1: 4 trials) and block 2 (day 2: 4 trials) in MWM training task was considered to be a measure of the learning process. The two-way ANOVA for the escape latency showed no significant effect of treatment ($F_{2,71} = 0.802$, $p = 0.453$), but revealed significant effect of block ($F_{1,71} = 29.741$, $p < 0.001$). There was no statistically significant interaction between treatment and block ($F_{2,71} = 1.315$, $p = 0.275$).The results of post hoc (Tukey test) analysis of differences for escape latency between block 1 and block 2 showed significant difference in all groups ($p < 0.05$). The results of paired *t* test analysis of differences for escape latency between block 1 and block 2 in the separate three groups are shown in Fig. 1; Supplementary Materials-1: Table S3.

The rats with GABAergic lesions exhibited a retention deficit 3 days later after training

1 h or 3 days after task acquisition, a probe test was performed to assess spatial memory. The spatial memory of the location of the hidden platform is indicated by preference for the test quadrant (Q_{test}) over the opposite quadrant (Q_{opp}). ANOVA was used to examine between-group differences on probe-test performance indices. One hour after training, the two-way ANOVA showed no significant effect of treatment ($F_{2,71} = 0.808$, $p = 0.450$), but showed significant effect of quadrant ($F_{1,71} = 179.274$, $p < 0.001$) and no significant interaction between treatment and quadrant ($F_{2,71} = 1.247$, $p = 0.294$). The results of post hoc (Tukey test) analysis of differences for time spent in Q_{test} and Q_{opp} showed a significant difference between quadrants in all groups ($p < 0.001$; Fig. 2). The results of paired *t* test analysis of differences for time spent in Q_{test} and Q_{opp} in the three groups separately 1 h after training are shown in Supplementary Materials-1: Table S4.

3 days after training, the two-way ANOVA did not reveal significant effect of group ($F_{2,71} = 0.787$, $p = 0.459$), but showed significant effect of quadrant ($F_{1,71} = 46.620$, $p < 0.001$) and interaction between group and quadrant ($F_{2,71} = 8.087$, $p < 0.001$; Fig. 3). The results of post hoc (Tukey's test) analysis of differences for time spent in Q_{test} and Q_{opp} showed no significant difference in GAT1-SAP lesion ($p > 0.05$) group and a significant difference between

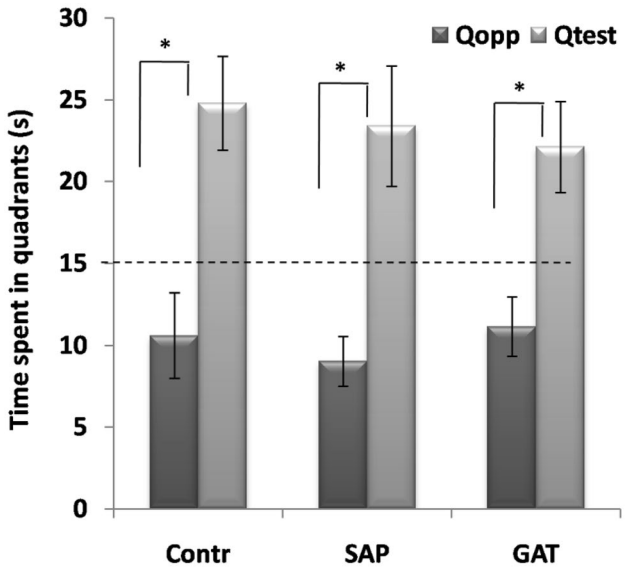

Fig. 2 Histograms showing probe-test performance of rats from different treatment groups 1 h after training. Spatial memory of the location of the hidden platform is indicated by preference for Q_{test} over Q_{opp}. Chance level (15 s)—dotted lines. During the probe test, trained rats of all groups spent significantly longer than chance in the test quadrant where the hidden platform was located in training trials and did not differ significantly among each other. The results of post hoc (Tukey's test after ANOVA) analysis of differences for time spent in Q_{test} and Q_{opp} showed a significant difference between quadrants in all groups ($*p < 0.001$)

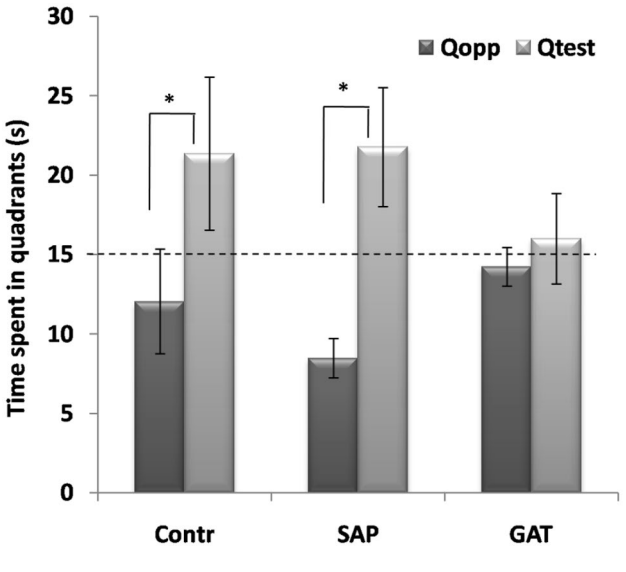

Fig. 3 Histograms showing probe-test performance of rats from different treatment groups 3 days after training. Long-term spatial memory of the location of the hidden platform is indicated by preference for Q_{test} over Q_{opp}. Chance level (15 s)—dotted lines. The results revealed that platform location was still being accessed by the control and 192 IgG-saporin-treated rats and thus indicated that selective lesion of cholinergic MS neurons did not affect spatial learning and memory in the MWM task. In marked contrast from cholinergic neurons, the present experiments indicate that rats with MS GABAergic lesions exhibited a retention deficit 3 days later after training. The results of post hoc (Tukey test after ANOVA) analysis of differences in time spent in Q_{test} and Q_{opp} 3 days after training showed no significant difference in the GAT1-SAP lesion ($p > 0.05$) group and a significant difference between quadrants of control and 192 IgG-saporin-treated groups ($*p < 0.000$)

quadrants of control and 192 IgG-saporin-treated groups ($p < 0.000$; see Supplementary Materials-1: Table S5).

To determine whether rats in each group learned the location of the hidden platform, the time spent in the target quadrant was compared to 15 s (the quadrant time expected from a random search of the water maze), using a one-sample *t* test. During the probe test which was performed 1 h after task acquisition, trained rats of all groups spent significantly longer than chance (15 s, dotted lines) in the test quadrant where the hidden platform was located in training trials (Contr: 24.792 ± 1.306, $t = 7.496$, $p < 0.001$; SAP: 23.381 ± 1.676, $t = 5.000$, $p < 0.001$, GAT: 22.124 ± 1.266, $t = 5.627$, $p < 0.001$). 3 days after task acquisition during the probe test, trained control and 192 IgG-saporin-treated rats spent significantly longer time than chance in the test quadrant (Contr: 21.360 ± 2.187, $t = 2.908$, $p = 0.008$; SAP: 21.782 ± 1.703, $t = 3.982$, $p = 0.001$). In contrast, in test quadrant GAT1-SAP-treated rats spent no longer than chance (16.0 ± 1.290, $t = 0.775$, $p = 0.446$). As all rats learned the location of the hidden platform during training trials, the results suggest that GAT1-SAP-treated rats could not remember the information acquired during training 3 days later.

Changes in neuroreceptor amounts

We studied the expression levels of cholinergic, GABAergic and glutamatergic receptors in the hippocampus of behaviorally characterized rats following selective lesions of different subsets of SH projections.

Immunostaining

Anti-GluR1 antibodies bound to a band of molecular weight 103 kDa, anti-NR2B stained a protein band with molecular weight 180 kDa, anti-α1 GABAA antibodies reacted with a protein band of molecular weight 52 kDa, anti-M1 muscarinic cholinoreceptor antibodies stained a protein band of molecular weight 50 kDa and antibodies against nicotinic acetylcholine receptor α7 bound to a band of molecular weight 56 Kda (Fig. 4). All of these weights corresponded to the expected size of the target proteins. Four standards (15, 30, 45, and 60 µg of total protein) were applied to each gel. For these standards, the optical densities of the immunostained bands (for GluR1, NR2B, α1 GABAA, muscarinic M1 or α7 nACh) were plotted against the amounts

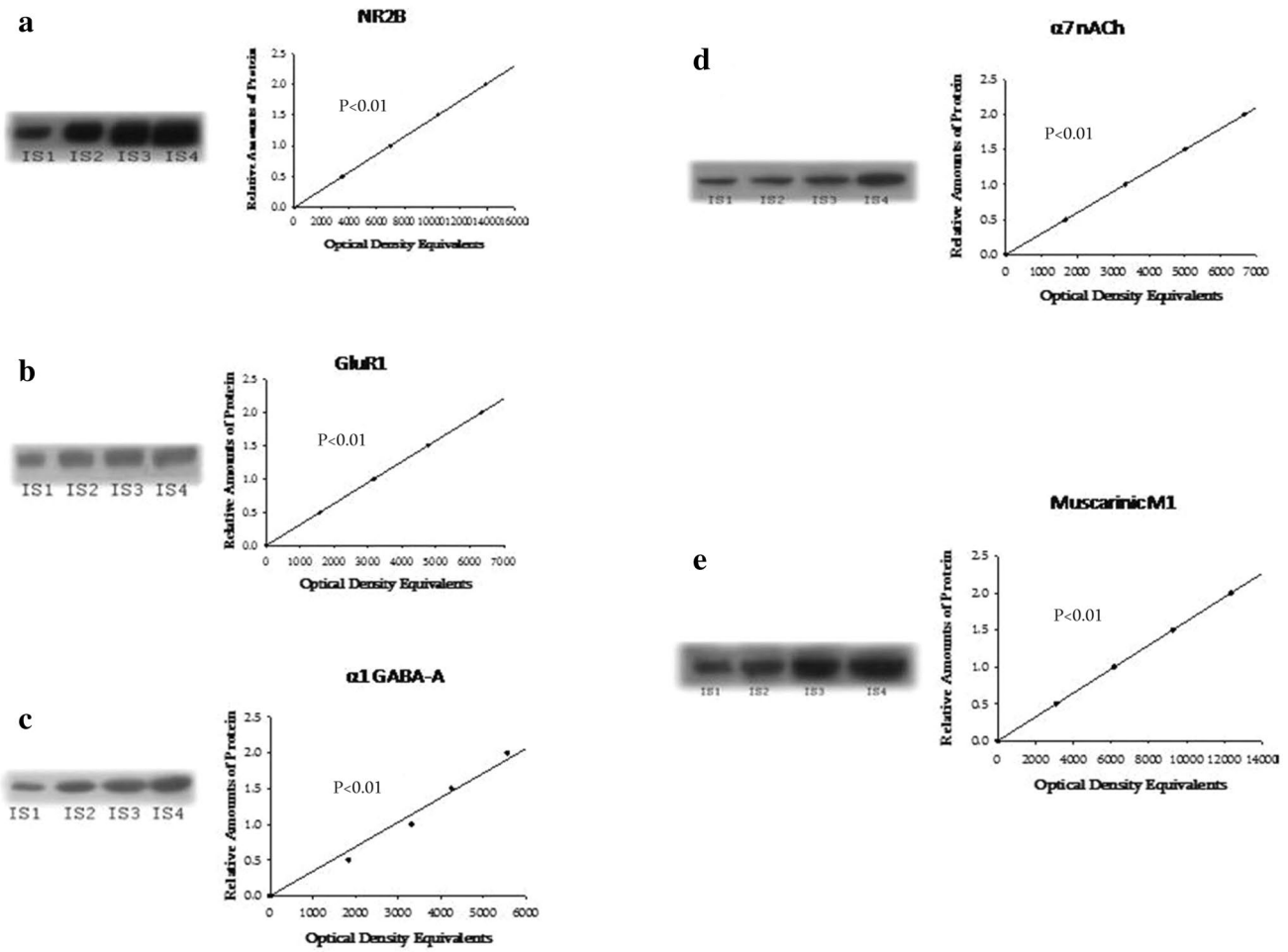

Fig. 4 Sample films of internal standards and calibration plots for **a** GluR1; **b** NR2B; **c** α1 GABAA; **d** muscarinic M1 and **e** α7 nACh. Bottom panels: sample radiographs; top panels: calibration plots (lines fitted by linear least-squares regression)

of protein; in all these standards, least-squares regression showed a significant fit to a straight line (Fig. 4).

NR2B subunit of NMDA receptor

The one-way ANOVA showed significant effect of treatment on the amount of NR2B ($F_{2.17} = 4.39$, $p = 0.032$). Planned comparisons revealed that the mean amount of NR2B subunit of NMDA receptor in the hippocampus was decreased significantly in the GAT1-SAP-treated group compared with the control ($t = 2.73$, $p = 0.021$, DF = 10). The difference between GAT1-SAP and 192 IgG-saporin-treated groups and between control and 192 IgG-saporin-treated groups was not significant (Fig. 5).

GluR1 subunit of AMPA receptor

The one-way ANOVA revealed significant effect of treatment on the amount of GluR1 ($F_{2.17} = 8.682$, $p = 0.003$). The mean amount of receptor was significantly lower in the GAT1-SAP-treated group as compared with the control ($t = 3.96$, $p = 0.003$, DF = 10) and as compared to the 192 IgG-saporin-treated group ($t = 2.99$, $p = 0.014$, DF = 10) (Fig. 6).

α1 subunit of GABAA receptor

The one-way ANOVA also showed significant effect of group ($F_{2.17} = 8.91$, $p = 0.003$). The mean amount of α1 subunit was significantly higher in the GAT1-SAP-treated group as compared to the control group ($t = 3.94$, $p = 0.003$, DF = 10) and as compared to the 192 IgG-saporin-treated group ($t = 2.75$, $p = 0.020$, DF = 10) (Fig. 7).

α7 nACh receptor

One-way ANOVA revealed a significant effect of treatment on the expression level of α7 subunit of nACh receptors

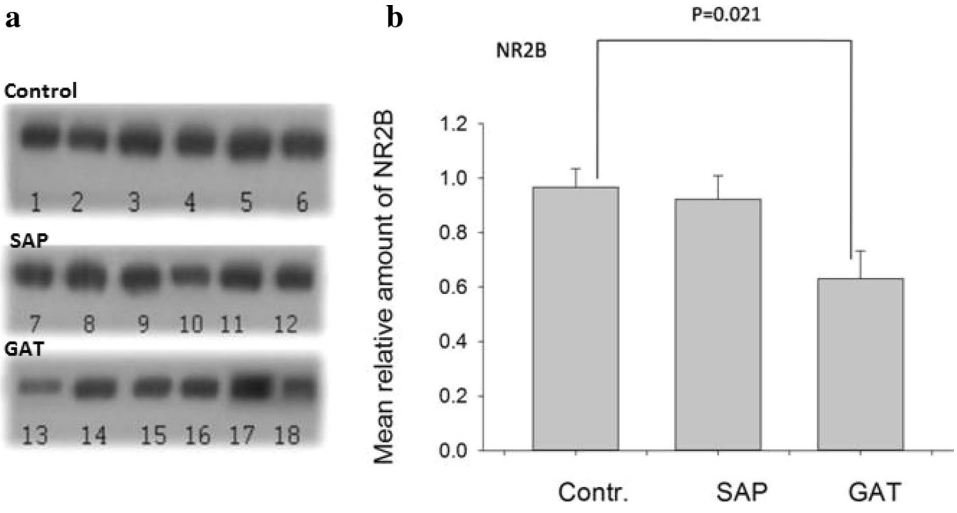

Fig. 5 Sample film (**a**) and mean levels (mean ± standard error of the mean) (**b**) of hippocampus NR2B in different groups of rats. Each lane corresponds to one sample. Lanes 1–6 are from control group; lanes 7–12 from 192 IgG-saporin-treated group and lanes 13–18 from GAT1-SAP-treated group. The gels contained irrelevant intercalated lanes, which have been excluded from the representative image. The full blot images for all neuroreceptors are provided as supplementary figures S4-S14. The same applies to the Figs. 6, 7, 8 and 9

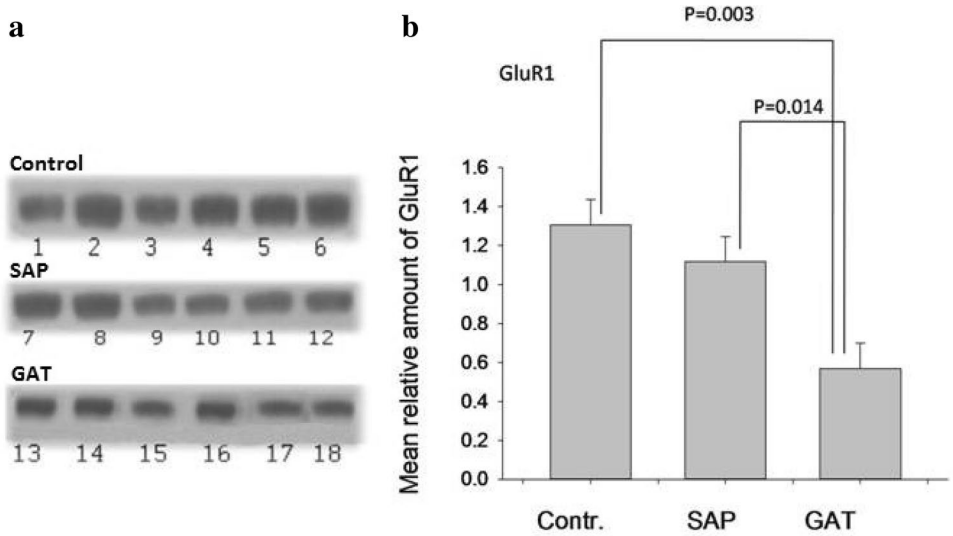

Fig. 6 Sample film (**a**) and mean levels (mean ± standard error of the mean) (**b**) of hippocampus GluR1 in different groups of rats. Each lane corresponds to one sample. Lanes 1–6 are from the control group; lanes 7–12 from the 192 IgG-saporin-treated group and lanes 13–18 from the GAT1-SAP-treated group

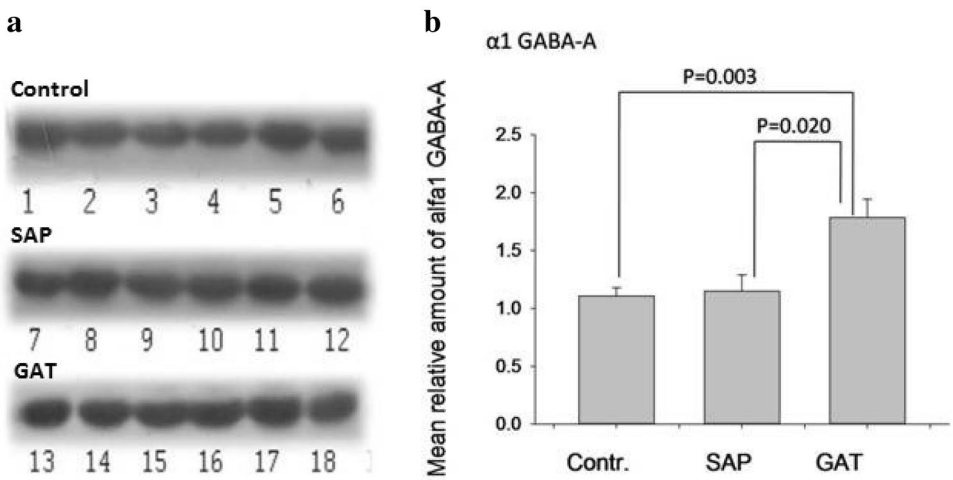

Fig. 7 Sample film (**a**) and mean levels (mean ± standard error of the mean) (**b**) of hippocampus α1 subunit in different groups of rats. Each lane corresponds to one sample. Lanes 1–6 are from the control group; lanes 7–12 from the 192 IgG-saporin group and lanes 13–18 from the GAT1-SAP-treated group

($F_{2.17}=8.88$, $p=0.003$). The mean level of α7 was significantly lower in the GAT1-SAP-treated group as compared to the control group ($t=2.75$, $p=0.020$, DF = 10) and as compared to the 192 IgG-saporin-treated group ($t=4.68$, $p=0.001$, DF = 10), (Fig. 8).

M1 receptor

There was a significant effect of treatment on the expression level of M1 cholinergic receptors in one-way ANOVA ($F_{2.17}=11.48$, $p=0.001$). The mean level of M1 receptors was significantly decreased in both treated groups as compared to the control group (GAT vs Contr—$t=4.02$, $p=0.002$, DF = 10 and SAP group vs Contr—$t=4.97$, $p=0.001$, DF = 10) (Fig. 9).

Discussion

The present results for the first time demonstrate that selective lesions of MS cholinergic and GABAergic neurons differentially affect spatial memory and the memory deficit after MS GABAergic lesion is paralleled with significant changes in the expression of hippocampal glutamate, GABA and acetylcholine receptor subunits.

This experiment compares two types of MS lesions: immunotoxin—192 IgG saporin infusions that mostly eliminate cholinergic neurons and GAT1-SAP infusions that predominantly affect the septal GABAergic neurons. The immunotoxic lesions of MS were verified by observing decreased ChAT and PV staining of the MS. ChAT and PV staining serves as a specific marker for cholinergic and GABAergic neurons. Microinjection of 192-IgG saporin into MS significantly reduces the number of AChE-sensitive neurons in the hippocampus; infusion of GAT1-saporin significantly reduced PV-positive cells in the MS and markers of cholinergic function in the hippocampus, but loss of

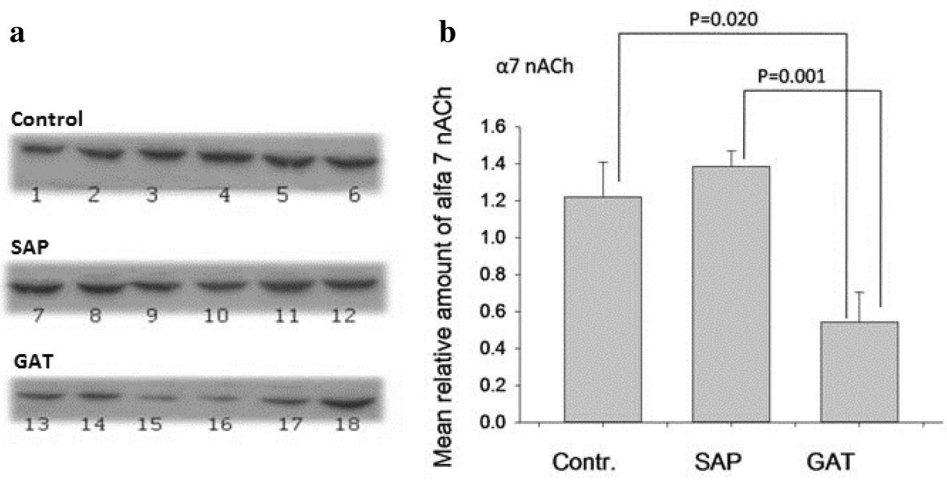

Fig. 8 Sample film (**a**) and mean levels (mean ± standard error of the mean) (**b**) of hippocampus α7nACh in different groups of rats. Each lane corresponds to one sample. Lanes 1–6 are from the control group; lanes 7–12 from the 192 IgG saporin group and lanes 13–18 from the GAT1-SAP group

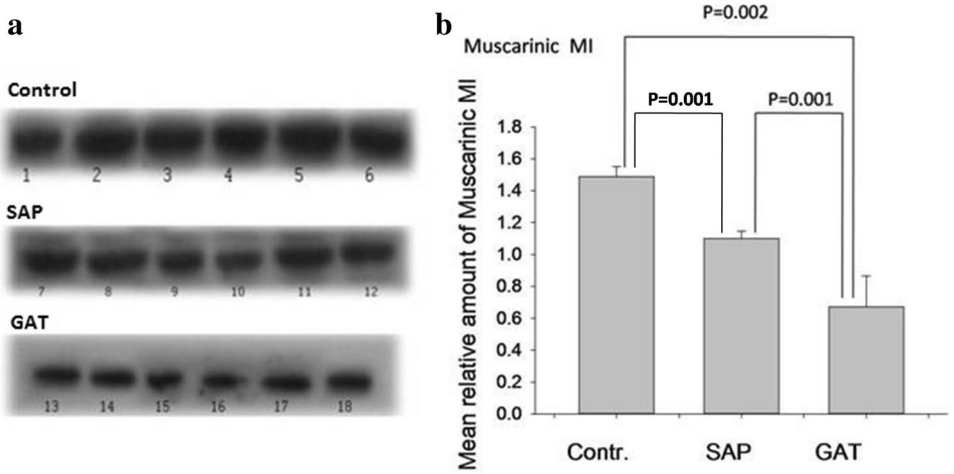

Fig. 9 Sample film (**a**) and mean levels (mean ± standard error of the mean) (**b**) of hippocampus M1 receptor in different groups of rats. Each lane corresponds to one sample. Lanes 1–6 are from the control group; lanes 7–12 from the 192 IgG saporin group and lanes 13–18 from the GAT1-SAP-treated group

AChE-sensitive neurons in the hippocampus was much less than that after 192 IgG-saporin lesions.

In behavioral experiments, we assessed acquisition of a spatial memory and its subsequent recall in the water maze. Following a 2-day training phase during which all groups improved their performance to comparable levels, retention was tested 1 h and 3 days later. The results showed that all rats exhibited a decreased latency to find the hidden platform across the eight training trials. These findings suggest that selective lesions of different subsets of SH projections did not prevent learning of a platform location. This absence of learning impairments after selective immunolesions is in line with previous studies showing no acquisition deficits in the water maze following either cholinergic or GABAergic lesions restricted to the septal region (Smith and Pang 2005; Lecourtier et al. 2011). During the probe test which was performed 1 h after task acquisition, trained rats of all groups spent significantly longer than chance in the test quadrant where the hidden platform was located in training trials and did not differ significantly among each other. The training was performed during two consecutive days to enable the establishment of a relatively robust memory, which can be recalled after 3 days of post-acquisition rest in rats with an intact brain.

The results obtained 3 days later, during the second probe test, showed that platform location was still being accessed by the control and 192 IgG-saporin-treated rats and thus indicated that selective lesion of cholinergic MS neurons did not affect spatial learning and memory in the MWM task. These findings suggest that SH cholinergic projections are not necessary for spatial memory. Previous studies, examining the effects of selective lesion of MS cholinergic neurons, have resulted in both impairments (Lamprea et al. 2003; Lehmann et al. 2003; Paban et al. 2005; Cai et al. 2012) and intact performance on a variety of spatial tasks (Baxter and Gallagher 1996; Winters and Dunnett 2004; Dashniani et al. 2015). However, the interpretation of the deficits after immunolesions consequent to selective loss of cholinergic MS neurons is sometimes questionable (see review: Parent and Baxter 2004).

Our Western blot analyses, conducted on the 14th post-lesion day, showed significant effect of 192 IgG-saporin treatment on the expression level of the M1 mACh receptors. The mean level of M1 receptors was significantly reduced in the treated group as compared to the control ones. Taking into account the results obtained in behavioral experiments on the same group of animals, it can be assumed that only a certain level of hippocampal M1 receptor function may be important for retention of MWM memory or these receptors are not at all crucial, at least, for a short post-acquisition time. 192 IgG-saporin treatment showed no significant effect on the amount of α1 subunit containing GABAA and α7 nACh receptors; no significant effect was revealed on the amount of glutamatergic NR2B subunit containing NMDA and GluR1 subunit containing AMPA receptors in the hippocampus. This result was unpredictable: it is shown that ACh can modulate glutamatergic transmission and plasticity in the hippocampus (Cheng and Yakel 2015). It can be assumed that the cholinergic deficiency after MS damage is temporarily compensated by hippocampal cholinergic interneurons, since the existence of such neurons in the hippocampus has been shown in numerous studies (Frotscher and Léránth 1985; Freund and Buzsáki 1996; Frotscher et al. 2000; Yi et al. 2015) and more significant changes in the function of the hippocampus develop with increasing time after injury (Paban et al. 2005; Lecourtier et al. 2011). Accordingly, it will be interesting to evaluate the expression level of hippocampal receptors together with the memory function at various post-lesion time intervals.

In marked contrast from cholinergic neurons, the present experiments indicate that rats with MS GABAergic lesions exhibited a retention deficit 3 days later after training. Behavioral analysis revealed that learning in MWM was not affected, but memory retention was significantly decreased. Moreover, our finding that selective lesion of GABAergic MS neurons affects probe-test performance, but not spatial, learning strongly suggests that GABAergic SH projections are involved specifically in the consolidation, but not in the acquisition of platform location. In the present study, immunohistological and Western blot analysis provide information about the state of the MS and hippocampus immediately after the second probe test. It is not known what neurobiological changes were present at the time of the first test, when spatial memory was intact in both lesion groups. It should be noted that no significant differences in behavioral results were observed in our pilot experiments conducted 10 days or 2 weeks after the MS lesion. These results show that the neurobiological changes described in this study evaluated 2 weeks after the lesion do not affect the learning process or short-term spatial memory.

Previous studies, including ours, discussed the significance of GABAergic SH projections in spatial memory (Dwyer et al. 2007; Pang et al. 2001, 2011; Lecourtier et al. 2011; Dashniani et al. 2015). Our results are in line with the results of Lecurtier et al. (2011), which showed that in the training phase there were no differences between the control and MS GABAergic lesioned rats: at the 1-day delay, all groups performed above chance and did not differ significantly among each other. At the 5-day delay, only rats with GABAergic lesions exhibited a retention deficit.

Taking into account the results of our behavioral experiments, special attention is deserved for the assessment of the effect of selective immunolesion of GABAergic MS neurons on the expression level of hippocampal receptors. It was revealed that the lesions of GABAergic MS neurons increase the expression of α1 subunit containing GABAA receptors

in the hippocampus. The enhanced GABAA receptor content in the hippocampus, revealed in the present study, may reflect a compensatory mechanism to maintain the functionality of this afferent connection. It has been shown that following lesion of the MS, extracellular GABA levels are reduced and GABAA receptor content increased in the hippocampus (Rodrıґguez et al. 2005).

There is possibility that the effects of the MS GABAergic lesion seen in our experiments may have altered the local circuit of the MS and the transmission between MS and hippocampus via non-GABAergic and non-cholinergic projections, for example via glutamatergic projections. Glutamatergic neurones have been described in this area, but their function remains unknown (Sotty et al. 2003). Therefore, we have studied also glutamate receptor expression. Unfortunately, there is no specific immunotoxin for selective lesions of glutamatergic neurons to follow this aspect further on. Less is known about the local circuitry of glutamatergic MS neurons; it has been suggested that they are both projection and interneurons (Gritti et al. 2006; Manseau et al. 2005). Some evidence indicates that intraseptal administration of glutamate receptor antagonists decreased hippocampal learning and memory (Huh et al. 2010; Henderson et al. 2010). In addition, SH cholinergic and GABAergic projections can alter glutamatergic transmission in the hippocampus. Kanju et al. (2012) demonstrated that septohippocampal cholinergic projections modulate hippocampal glutamatergic synaptic transmission and, thereby, alter synaptic plasticity mechanisms required for learning and memory. It has been shown that up-regulation of α7 nAChRs on presynaptic sites of glutamatergic terminals increases glutamate release (Perry et al. 2000) and facilitates NMDA-dependent glutamatergic responses of hippocampal pyramidal neurons (Kuryatov et al. 2005).

Our Western blot results showed clearly that there was a significant decline in hippocampal NR2B and GluR1 protein levels after GAT1-SAP administration to the MS, suggesting that selective GABAergic lesions of the MS affect glutamatergic transmission in the hippocampus. The significance of NR2B subunit of NMDA receptor in the consolidation of long-term spatial memory has been indicated in some previous studies. Ge et al. (2010), using a MWM task, showed a functional requirement of NR2B subunits of NMDA receptor in the consolidation of long-term spatial memory. The role of GluR1-containing AMPA receptors in synaptic plasticity has been extensively studied. It has been demonstrated that the regulation of AMPA receptor cycling and surface trafficking play a critical role in the induction of long-term potentiation (LTP) in hippocampal neurons (Kakegawa et al. 2004).

Regarding the hippocampal cholinergic transmission, our Western blot analyses showed significant decline in cholinergic α7 nACh and M1receptors expression after GAT1-SAP administration to the MS. It should be noted that both, mACh and nACh receptors can modulate synaptic transmission and plasticity in the hippocampus (Shen and Yakel 2012; Takata et al. 2011). It has been shown that when α7 nACh receptors agonists were both perfused, the activation of α7 nACh receptors facilitated the induction of LTP in the CA1 hippocampal region (Hunter et al. 1994; Fujii et al. 1999; Cheng and Yakel 2015). The α7 nACh receptors-mediated enhancement of LTP was due to its ability to increase presynaptic release of glutamate and postsynaptic depolarization. Fernandez de Sevilla et al. (2008, 2010) showed a long-term enhancement of both AMPA- and NMDA receptors-mediated transmission induced by a brief local application of ACh on the apical dendrites of CA1 pyramidal cells was dependent on the activation of M1 mACh receptors. Taking into account the existing as well as our results, it can be assumed that selective GABAergic MS damage may affect hippocampal-dependent spatial memory, by modulating glutamatergic as well as cholinergic transmission in the hippocampus. The process which determines the alteration in the expression of the hippocampal receptors following MS GABAergic lesions is the subject of further research.

Conclusion

In summary, our findings for the first time demonstrated two important points. First, selective lesions of MS cholinergic and GABAergic neurons differentially affect spatial memory: the immunolesion of GABAergic MS neurons causes impairment of hippocampal-dependent spatial memory; damage of SH cholinergic projections is insufficient for impairment of spatial memory, at least for the early post-acquisition time point. Second, selective GABAergic lesions of the MS affect hippocampal-dependent spatial memory probably through modulation of glutamatergic as well cholinergic and GABAergic receptor expression in the hippocampus. Understanding of the mechanisms by which SH system can modulate memory, and the aspects of memory for which it may be essential, can lead to new strategies for memory improvement in patients with neurodegenerative disease, as well as to a better understanding of the fundamental neurobiology of memory.

Author contributions MD and MB contributed to the conception and design; MD, MB and RS contributed to analysis and interpretation of data in the manuscript; GB, LK, NC, MC, and MK managed the data collection. Statistical analysis was done by MD, RS and MB. RS and TN revised the manuscript. All authors declare the responsibility for every aspect of the work. All authors read and approved the final manuscript.

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Availability of data and material All primary data are provided in Supplementary Material 2.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethical approval All experimental procedures were conducted in accordance with the European Communities Council Directive Guidelines for the care and use of laboratory animals (2010/63/EU—European Commission) and approved by the animal care and use committee at the I. Beritashvili Center of Experimental Biomedicine.

Consent for publication All co-authors have agreed to the submission of the final manuscript.

Code availability Not applicable.

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