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# SPATIAL LEARNING-INDUCED ACCUMULATION OF AGMATINE AND GLUTAMATE AT HIPPOCAMPAL CA1 SYNAPTIC TERMINALS

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**Abstract—Agmatine, the decarboxylated metabolite of L-arginine, is considered to be a novel putative neurotransmitter. Recent studies have demonstrated that endogenous agmatine may directly participate in the processes of spatial learning and memory. Agmatine-immunoreactivity has been observed within synaptic terminals of asymmetric excitatory synapses in the hippocampal CA1 stratum radiatum (SR), suggesting that agmatine may be colocalized with glutamate. In the present study we demonstrate, using immunofluorescence confocal microscopy, that agmatine is colocalized with glutamate within CA1–CA3 hippocampal pyramidal cell bodies, in young Sprague–Dawley rats. Subcellular investigation, using postembedding electron microscopy-immunogold cytochemistry, has also revealed that agmatine is colocalized with glutamate in most synaptic terminals in the SR region of CA1. Ninety-seven percent of all agmatinergic profiles were found to contain glutamate, and 92% of all glutamatergic profiles contained agmatine ($n$=6; 300 terminals). Alterations in colocalized agmatine and glutamate levels in the SR synaptic terminals, following 4 days Morris water maze training, were also investigated. Compared with swim only control rats, water maze-trained rats had statistically significant increases in both agmatine (78%; $P<0.01$) and glutamate (41%; $P<0.05$) levels within SR terminals synapsing onto CA1 dendrites. These findings provide the first evidence that agmatine and glutamate are colocalized in synaptic terminals in the hippocampal CA1 region, and may co-participate in spatial learning and memory processing.** 

**Key words: CA1, agmatine, glutamate, water-maze, spatial learning, immunogold.**

Agmatine is a decarboxylated metabolite of L-arginine. It is widely prevalent in the mammalian brain and other organs (Raasch et al., 1995). Agmatine is considered a novel putative neurotransmitter in terms of its synthesis, storage, inactivation, degradation, release, and binding properties (for a review see Reis and Regunathan, 2000). Its concentration in the brain is similar to that of classic neurotransmitters (Li et al., 1994). In the hippocampus, agmatine is primarily localized in the CA1 and CA3 pyramidal cell bodies and the granule cells of the dentate gyrus (DG). Diffuse agmatine-immunoreactivity (IR) is also present throughout the strata oriens, stratum radiatum (SR), and stratum lacunosum-moleculare (Reis et al., 1998).

Several studies have investigated the role of endogenous agmatine in learning and memory. It has been shown that spatial reference memory (e.g. rats are trained to find a hidden platform in a fixed position in the water maze (WM) over multiple daily training sessions) requires neural activity in the CA1 sub-region of the hippocampus (Jo et al., 2007). Liu et al. (2008a) reported that WM training (eight trials per day for four consecutive days) resulted in 60–80% increases in the agmatine levels in the hippocampal CA1 region in young adult rats, when gross tissue content was assessed by liquid chromatography/mass spectrometry (LC/MS). Using quantitative immunogold-labeling coupled with high-resolution low-temperature embedding electron microscopy (EM) techniques, Leitch et al. (2011) reported that agmatine levels in the CA1 SR synaptic terminals were increased by approximately 85% in WM-trained rats compared with swim only (SW) control rats. Liu et al. (2009) demonstrated that T-maze training-induced increases in agmatine levels (gross tissue content) in the perirhinal and prefrontal cortices, which positively correlated with animals' performance (the number of correct responses) in the T-maze task. Collectively, these studies provide strong evidence that endogenous agmatine may directly participate in the processes of learning and memory, and that learning-induced increase in agmatine may have important functional significance.

In the SR of hippocampal CA1 region, agmatine-IR is primarily localized within synaptic terminals which form asymmetrical synapses with dendrites and dendritic spines of pyramidal cells (Reis et al., 1998; Leitch et al., 2011). Because these synapses are similar to the type of contacts associated with the N-methyl-D-aspartic acid (NMDA) receptor (Siegel et al., 1994), it has been suggested that agmatine may be co-stored and co-released with L-glutamate, the principal excitatory transmitter of hippocampal pyramidal cells (Reis et al., 1998).

The first aim of the study was to determine whether agmatine is colocalized with L-glutamate at hippocampal pyramidal cells, using immunofluorescence confocal microscopy. EM-immunogold cytochemistry (EM-ICC) was then used to quantify the extent of colocalization at CA1 SR synaptic terminals at the subcellular level. The second aim of the study was to investigate whether the changes in colocalized agmatine and glutamate levels occur in the CA1 SR synaptic terminals following WM training, as

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*Abbreviations:* ADC, agmatine decarboxylase; BSA, bovine serum albumin; EM, electron microscopy; IR, immunoreactivity; NO, nitric oxide; PB, phosphate buffer; PBS, phosphate-buffered saline; LTP, long-term potentiation; SC, Schaffer collateral; SR, stratum radiatum; SW, swim only; WM, water maze.

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Leitch et al. (2011) have previously demonstrated the learning-induced changes in agmatine at these profiles.

## EXPERIMENTAL PROCEDURES

### Animals

Male Sprague–Dawley rats were raised from breeding stock obtained from University of Otago Hercus Taieri Resource Unit. Adult male rats (320–350 g) were housed two per cage ($21\times34\times18$ $cm^3$) with 12-h light/dark cycle (lights on at 7 A.M.) and were provided *ad libitum* access to food and water. The University of Otago Committee on Ethics in the Care and Use of Laboratory Animals approved all animal protocols used, and every attempt was made to reduce the number of animals used and to minimize their sufferings.

### Behavioral test

The behavioral study was conducted according to the method described in Leitch et al. (2011). Briefly, tests were carried out in a windowless room with three clear and one red 75 W bulbs mounted on the ceiling. A video camera was mounted at ceiling height in the center of the room and used for recording the performance during the experimental period. A radio speaker was located adjacent to the video camera at ceiling height to provide background masking noise. The WM pool, comprised a black circular tank (150 cm in diameter and 45 cm in height), was filled with water to a depth of 25 cm and maintained at a temperature of $25\pm1$ °C (Liu et al., 2008a,b; Leitch et al., 2011). Four points around the edge of the poor were designated as North, South, East and West to allow the apparatus to be divided into four corresponding quadrants, such as Northeast, Northwest, Southeast and Southwest. The extramaze cues (the laboratory furniture, lights and several prominent visual features on the walls, as well as the location of the experimenter) were held constant throughout the entire study.

After a 5-day period of acclimatization, the rats were divided into WM-trained ($n=3$) and SW control ($n=3$) groups. The WM rats were trained to find a hidden escape platform that was located in the center of the Southeast quadrant and submerged 2 cm below the water surface. There were eight trials per day with 120 s of intertrial intervals for four consecutive days. On each trial, the rat was placed into the pool facing toward the wall, and was allowed to swim freely in search of the escape platform. If the rat found the platform, it was allowed to remain on the platform for 10 s before being removed and placed in a holding box. If the rat was unsuccessful in locating the platform within 120 s, it was immediately placed on the platform for 10 s and was then put in the holding box. During the 120-s intertrial interval, the rat was dried off, warmed, and kept in the holding box. Starting locations (North, South, West, East) were pseudorandomly selected. The SW rats were placed in the pool and allowed to swim freely with no platform presented. For each SW rat, the duration of swimming in each trial was matched to its counterpart in the WM group. After completion of the WM test, the distance the rats swam from the starting point to reach the platform (path length) was analyzed from a computerized tracking system (HVS, 2020). Animals in the WM group showed a rapid decrease in path length (averaged across eight trials for each day) across the 4 days of training ($5.99\pm0.98$ m on day 1 and $2.23\pm0.58$ m on day 4). The mean path length in the SW group was $5.32\pm0.71$ m and $2.09\pm0.73$ m on day 1 and day 4, respectively.

### Antibodies, controls and specificity tests

The primary antibodies used to detect agmatine were commercially available affinity-purified polyclonals obtained from two sources, Gemacbio (AP124) and Millipore (AB1568-2000T). The antibodies were raised in rabbits after immunizing with a conjugate agmatine-glutaraldehyde-bovine serum albumin (BSA) carrier. The procedures used to obtain these polyclonal antisera and to test their specificities are provided in the manufacturers' product data sheets. Both anti-agmatine antibodies tested produced identical results in immunohistochemical applications. The anti-glutamate antibody was a mouse monoclonal (G9282, Sigma) which binds specifically with L-glutamate. It was derived from the GLU-4 hybridoma which was produced by the fusion of mouse splenocytes and myeloma cells of an immunized mouse. The immunogen used to produce the antibody was L-glutamic acid conjugated to a protein carrier with glutaraldehyde. The pattern of labeling obtained with this mouse monoclonal antibody was identical to that produced by rabbit polyclonal anti-glutamate antibodies published in previous studies (Richardson and Leitch, 2005; Kolodziejczyk et al., 2008). In addition, similar staining patterns for the glutamate mouse monoclonal antibody was produced using vesicular glutamate transporter (vGlut1; MM-0016-6P-FS, Medimabs) in immunohistochemical procedures.

Optimal antibody concentrations for immunofluorescence confocal microscopy and EM-immunocytochemistry were individually determined for each antibody using a serial double-dilution method, where a wide range of dilutions were tested for each antibody on either side of the supplier's recommended dilutions.

To confirm the specificity of the batches of antibodies used in this study, sections were incubated with a primary antibody solution that was first incubated for 2–3 h at room temperature with an excess (10 $\mu$g peptide per 1 $\mu$g antibody) of the supplier's peptide (AG 124) against which the antibody was generated. To test the specificity of the secondary antibodies, and check for background staining, sections were treated identically to experimental sections except for the omission of the primary antibodies.

In immunohistochemical procedures, the primary and secondary antibodies were also tested for cross-reactivity. Sections were incubated with either rabbit polyclonal anti-agmatine antibody or the mouse monoclonal anti-glutamate antibody, followed by a mixture of both secondaries (Alexa 488 goat anti-rabbit antibody and Alexa 568 goat anti-mouse antibody).

### Immunofluorescence confocal microscopy

Rats were deeply anesthetized with sodium pentobarbital solution (Nembutal 60 mg/kg i.p.) and perfused transcardially with 5% heparin in phosphate-buffered saline (PBS) pH 7.4, followed by 4% paraformaldehyde (PFA) with 0.1% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (PB) pH 7.4. The brains were extracted from skulls, cryoprotected with increasing concentrations (10%, 20%, 30%) of sucrose, and were coronally sectioned into 30-$\mu$m thick sections with sledge microtome (Leitz 1300, Leica Microsystems, Wetzlar, Germany). Sections were treated with 1% sodium borohydrate for 30 min and with blocking solution (4% normal goat serum, 0.1% BSA and 0.3% TritonX-100 in PBS) for 2 h. Sections were incubated in anti-agmatine antibody (1:500) and mouse monoclonal anti-glutamate (1:20,000) for 72 h, followed by overnight incubation in Alexa 488 goat anti-rabbit antibody (Invitrogen, Yaphank, NY, USA; 1:1000) and Alexa 568 goat anti-mouse antibody (Invitrogen, Yaphank, NY, USA; 1:1000). Sections were mounted on glass slides, dried and covered with DABCO-Glycergel on coverglass.

### EM immunocytochemistry

Three pairs of Sprague–Dawley rats were deeply anesthetized with sodium pentobarbital solution (Nembutal 60 mg/kg i.p.) and perfused transcardially with 5% heparin in PBS pH 7.4, followed by 2% PFA with 2.5% glutaraldehyde in 0.1 M PB pH 7.4. Brains were removed from skulls, fixed in fresh fixative for 3 h at room temperature and were processed for EM immunocytochemistry according to the methods of Leitch et al. (2009a–c, 2011). The hippocampus was isolated, washed in PB and was vibratomed into 200 $\mu$m thick sections (HA752 vibroslice tissue cutter, Campden instruments Ltd., UK). Dorsal hippocampal sections were