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REGION-SPECIFIC CHANGES IN PRESYNAPTIC AGMATINE AND GLUTAMATE LEVELS IN THE AGED RAT BRAIN

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Abstract—During the normal aging process, the brain undergoes a range of biochemical and structural alterations, which may contribute to deterioration of sensory and cognitive functions. Age-related deficits are associated with altered efficacy of synaptic neurotransmission. Emerging evidence indicates that levels of agmatine, a putative neurotransmitter in the mammalian brain, are altered in a region-specific manner during the aging process. The gross tissue content of agmatine in the prefrontal cortex (PFC) of aged rat brains is decreased whereas levels in the temporal cortex (TE) are increased. However, it is not known whether these changes in gross tissue levels are also mirrored by changes in agmatine levels at synapses and thus could potentially contribute to altered synaptic function with age. In the present study, agmatine levels in presynaptic terminals in the PFC and TE regions (300 terminals/region) of young (3 month; $n = 3$) and aged (24 month; $n = 3$) brains of male Sprague–Dawley rats were compared using quantitative post-embedding immunogold electron-microscopy. Presynaptic levels of agmatine were significantly increased in the TE region (60%; $p < 0.001$) of aged rats compared to young rats, however no significant differences were detected in synaptic levels in the PFC region. Double immunogold labeling indicated that agmatine and glutamate were co-localized in the same synaptic terminals, and quantitative analyses revealed significantly reduced glutamate levels in agmatine-immunopositive synaptic terminals in both regions in aged rats compared to young animals. This study, for the first time, demonstrates differential effects of aging on agmatine and glutamate in the presynaptic terminals of PFC and TE. Future research is required to understand the functional significance of these changes and the underlying mechanisms.

Key words: aging, agmatine, glutamate, immunogold, prefrontal cortex, temporal cortex.

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Abbreviations: BSA, bovine serum albumin; EM ICC, electron microscopic immunocytochemistry; EM, electron microscopy; NO, nitric oxide; NOS, nitric oxide synthase; PB, phosphate buffer; PFC, prefrontal cortex; TE, temporal cortex.

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INTRODUCTION

Agmatine, a metabolite of L-arginine by arginine decarboxylase, is widely distributed in mammalian brains. It is considered to be a novel putative neurotransmitter, because it is synthesized in the brain, stored in synaptic vesicles, accumulated by uptake, released by depolarization, and inactivated by agmatinase (for reviews see Reis and Regunathan, 1998a,b, 2000; Piletz et al., 2013). There is also evidence suggesting that endogenous agmatine participates in learning and memory processes (Liu et al., 2008b, 2009; Leitch et al., 2011; Seo et al., 2011; Rushaidhi et al., 2013). Moreover, agmatine regulates the production of nitric oxide (NO) and polyamines; hence it has a central role in regulating arginine metabolism (Piletz et al., 2013).

Levels of agmatine in the brain are altered with age. For example, Raasch et al. (1995) reported a reduction in cortical agmatine levels in 24-month-old male Long Evans rats when compared to 3- and 14-month-old rats (about 35% and 50% reduction, respectively). We previously investigated how the gross tissue content of agmatine changed with age in the prefrontal cortex (PFC), hippocampus and parahippocampal region of male Sprague–Dawley (SD) rats. Interestingly, there was a 50% reduction in agmatine concentration in the PFC in the aged rats (24 months) when compared to the young rats (4 months) regardless of animals' behavioral experience (Liu et al., 2008a; Gupta et al., 2012). In contrast to the PFC, other brain regions of aged rats, including the parahippocampal region and temporal cortex (TE, an auditory cortex), showed 60–180% increases in agmatine levels when compared to young rats (Liu et al., 2008a; Gupta et al., 2012).

While these studies have shown altered agmatine levels with age in a region-specific manner, the underlying mechanisms and functional significance of these diverse changes are not fully understood at present. It is of interest to note that the total NO synthase (NOS) activity was significantly increased with age in the PFC and TE, and there were significant negative and positive correlations between levels of agmatine and NOS activity in the PFC and TE, respectively (Gupta et al., 2012). Since agmatine regulates NO production by inhibiting neuronal and inducible NOS (for reviews see Reis and Regunathan, 2000; Piletz et al., 2013), higher levels of agmatine in the TE might be a compensatory mechanism to control age-related increase in NOS activity (i.e. to maintain NO at physiological levels), whereas lower levels of agmatine in the PFC might fail to do so. Rushaidhi

et al. (2012) revealed reduced agmatine concentrations in whole-tissue extracts and in synaptoneurosomes (a subcellular preparation enriched for pre and postsynaptic tissue) in the aged PFC. Given the role of endogenous agmatine in learning and memory (Liu et al., 2008b, 2009; Leitch et al., 2011; Seo et al., 2011; Rushaidhi et al., 2013), decreased levels of agmatine may lead to altered neurotransmission in the PFC. However, it is unclear how the agmatine level changes with age in presynaptic terminals, where it could impact on synaptic function.

At synapses, agmatine evokes a non-competitive voltage- and concentration-dependent block of the N-methyl-D-aspartate (NMDA) receptors (Reis and Regunathan, 2000; Halaris and Plietz, 2007). Since agmatine is co-localized with the principal excitatory neurotransmitter glutamate, at synapses (Reis et al., 1998; Seo et al., 2011), it may have an important role in controlling glutamate-mediated excitatory moiety. Hence, the aim of the present study was to determine if age-related changes in agmatine in PFC and TE, previously reported in whole-tissue extracts, are mirrored by changes at excitatory synapses, using quantitative post-embedding immunogold electron microscopy (EM). Furthermore, given the co-localization of agmatine and glutamate in presynaptic terminals, we also investigated where glutamate levels are altered in the synaptic terminals of agmatine-immunopositive neurons in PFC and TE regions in aged animals compared to young ones.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley (SD) rats, young (3 months, $n = 3$) and aged (24 months, $n = 3$) were housed individually ($21 \times 33 \times 18$ cm^3) under specific pathogen-free environmental conditions, maintained on a 12-h light–dark cycle (lights on 7 a.m.) and provided *ad lib* access to food and water. The health condition (e.g., body weight, eyes, teeth, fur, skin, feet, urine and general behavior) of aged animals was regularly monitored by animal technicians and a consultant veterinarian. Only animals showing good health were used for the study. All experimental procedures were carried out in accordance with the regulations of the University of Otago Committee on Ethics in the Care and Use of Laboratory Animals. Every attempt was made to limit the number of animals used and to minimize their suffering.

EM immunocytochemistry

Rats were deeply anesthetized with sodium pentobarbital solution (Nembutal 60 mg/kg i.p.) and transcardially perfused with 5% heparin in phosphate-buffered saline (PBS) pH 7.4 followed by 4.0% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (PB) pH 7.4. After perfuse-fixation, brains were removed from the skulls, postfixed in fresh fixative for 3 h at room temperature, then processed for electron microscopic immunocytochemistry (EM ICC) according to well established methods published by our lab (Leitch et al., 2009a,b,c, 2011; Seo et al., 2011; Seo and Leitch, 2015; Trotman et al., 2014). The PFC (Fig. 1A) and TE (Fig. 2A) were dissected out, washed in PB, and sliced into 250-μm-thick coronal sections under PB using a vibratome (HA752 Vibroslice tissue cutter, Campden instruments, Loughborough, UK). Great care was taken to ensure that all tissue sections were taken from the same regions in all rats (Figs. 1A and 2A). Tissue sections were washed in PB overnight, and then placed into sucrose solutions of increasing concentration for cryo-protection. Coronal sections were impact frozen onto copper blocks cooled in liquid N2 (Leica Microsystems, EM MM80E, Wetzlar, Germany) for freeze substitution (Leica Microsystems, EM AFS2, Wetzlar, Germany) and low-temperature embedding in Lowicryl HM 20 resin (Chemische Werke Lowi, Waldkraiburg, Germany) according to Leitch et al. (2011). For each brain region, 70-nm-thick serial coronal sections containing the whole selected brain region were cut by an ultramicrotome and picked up on formvar-coated nickel grids. As the blocs were cut en face, all cortical cell layers were represented in each coronal section.

Postembedding immunocytochemistry was carried out on 70-nm-thick serial coronal sections on nickel grids (Richardson and Leitch, 2002, 2005; Seo et al., 2011). For single labeling with agmatine, sections were first floated face downward on droplets of 5% normal goat serum in bovine serum albumin (BSA)/Tris buffer, pH 7.4 for 30 min, then incubated in rabbit polyclonal anti-agmatine antibody (Gemacbio, France), at a dilution of 1:100 at 4 °C overnight. After thorough washing in BSA/Tris buffer, pH 7.4, sections were incubated with secondary antibody (British BioCell, Cardiff, UK 1:20) coupled to 15-nm gold particles then washed in ultra-pure water and contrasted with saturated aqueous uranyl acetate followed by lead citrate for EM analysis. Sections were also double labeled to detect both agmatine and glutamate as previously described (Seo et al., 2011) Briefly, after the sections have been incubated with agmatine and its secondary antibody as described above, grids were incubated in mouse monoclonal anti-glutamate antibody (G9282, sigma) at a dilution of 1:1000 overnight, then secondary antibody (British BioCell, Cardiff, UK 1:20) coupled to 5-nm gold particles. Sections were then washed in ultra-pure water and contrasted with saturated aqueous uranyl acetate followed by lead citrate for EM analysis. Sections were imaged with Philips EM 410LS EM with megaview two digital imaging camera and analysis software ITEM (Soft Imaging System, Munster, Germany). Immuno-labeled sections were coded and randomly selected so that the operator was blind to the experimental treatment conditions.

Antibodies, controls and specificity tests

The primary antibody used to detect agmatine was a commercially available affinity-purified polyclonal obtained from Gemachio (France). The antibody was raised in rabbits after immunization with synthetic agmatine conjugated to the protein carrier BSA with glutaraldehyde. The anti-glutamate primary antibody was a mouse monoclonal (G9282, Sigma), which binds specifically with L-glutamate. It was derived from the GLU-4 hybridoma that was produced by the fusion of