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SHORT COMMUNICATION

Synaptic localisation of agmatinase in rat cerebral cortex revealed by virtual pre-embedding

V. I. Madai · W. C. Poller · D. Peters · J. Berger · K. Paliege · R. Bernard · R. W. Veh · G. Laube

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Abstract Light microscopic evidence suggested a synaptic role for agmatinase, an enzyme capable of inactivating the putative neurotransmitter and endogenous anti-depressant agmatine. Using electron microscopy and an alternative pre-embedding approach referred to as virtual pre-embedding, agmatinase was localised pre- and postsynaptically, to dendritic spines, spine and non-spine terminals, and dendritic profiles. In dendritic spines, labelling displayed a tendency towards the postsynaptic density. These results further strengthen a synaptic role for agmatine and strongly suggest a regulatory role for synaptically expressed agmatinase.

Keywords Agmatinase · Agmatine · Neurotransmission · Pre-embedding · Mood disorders

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V. I. Madai · W. C. Poller · D. Peters · J. Berger · K. Paliege · R. Bernard · R. W. Veh · G. Laube (✉)
Institute for Integrative Neuroanatomy,
Charité—Universitätsmedizin Berlin,
Philippstr. 12, 10115 Berlin, Germany
e-mail: gregor.laube@charite.de

V. I. Madai · R. Bernard
Center for Stroke Research Berlin (CSB),
Charité—Universitätsmedizin Berlin, Berlin, Germany

W. C. Poller
Department of Cardiology, Charité—Universitätsmedizin Berlin,
Berlin, Germany

K. Paliege
Department of Otolaryngology, Head and Neck Surgery at UKB,
Unfallkrankenhaus Berlin, Berlin, Germany

Introduction

Agmatinase (Agm), an arginase family enzyme converting agmatine to putrescine, is widely expressed in the mammalian brain (Bernstein et al. 2011). It is not known, however, whether putrescine derived from agmatine degradation is used to also fuel an ornithine decarboxylase-independent polyamine synthesis (Gilad et al. 1996). Both pathways for putrescine synthesis are intimately related by using the common substrate arginine, similar transport systems and regulatory interactions of agmatine with polyamine synthesis (Agostinelli et al. 2010). Changes in polyamine metabolism have been observed in stroke (Kim et al. 2004; Wang et al. 2010) and several other neuropathological conditions (Lewandowski et al. 2010; Seidl et al. 1996). Evidence suggests that agmatine plays a role as a neurotransmitter (Reis and Regunathan 2000). Consistently, agmatine was released from purified spinal nerve terminals upon depolarisation (Goracke-Postle et al. 2007). Thus, Agm may serve to locally inactivate neuromodulatory effects of synaptically released agmatine. The ultrastructural localisation of Agm may therefore provide further evidence for a synaptic role for agmatine and help to identify synapse populations as potential targets for pharmacological intervention. So far, the cellular expression patterns of Agm in rat and healthy human brain (Bernstein et al. 2011), as well as in human brains of individuals suffering from mood disorders (Bernstein et al. 2012), have been described. In the rat, besides neuronal cell bodies, a diffuse Agm labelling was observed in several neuropil areas, including the cerebral cortex. To unequivocally localise Agm to synapses, we used standard diaminobenzidine (DAB)-based immunocytochemistry and a novel, sensitive hapten-based pre-embedding technique for tissue sections. This method is here referred to as

"virtual pre-embedding" (VirP), since the marker used for visualisation is not introduced during pre-embedding but in a subsequent post-embedding step.

Materials and methods

For a detailed protocol and discussion of the VirP method, see supplement S1.

The generation, purification and characterisation of the monospecific antibody against agmatinase have been described previously (Bernstein et al. 2011).

Preparation of rat tissue

All animal experiments were conducted in accordance with the guidelines of the European Communities Council directive 86/609/EEC and were approved by the Regional Berlin Animals Ethics Committee (LaGeSo No. G 0168/01). Rat brain tissue was prepared as described previously (Krauss et al. 2006).

Preparation of tetramethylrhodamine-tyramide

Tetramethylrhodamine-tyramide (TMRT) was synthesised as described elsewhere (Hopman et al. 1998) with minor modifications. Briefly, a solution of tyramine-HCL in dimethylformamide (DMF), containing 0.9% triethylamine to deprotonate the amino group of tyramine, was mixed with a solution of the N-hydroxysuccinimide ester of tetramethylrhodamine (TMR) in DMF in an equimolar ratio.

ABC/DAB procedure for light (LM) and electron microscopy (EM)

Free floating vibratome sections were treated similarly for light and electron microscopy, as previously described (Krauss et al. 2007). For EM, Triton X-100 was used only during pre-incubation with blocking protein for 30 min.

Virtual pre-embedding procedure

With virtual pre-embedding (VirP), haptens are used in a combination of pre- and post-embedding procedures (Humbel et al. 1998). Haptens are deposited in close vicinity of the antigen by catalysed reporter deposition (CARD) in a pre-embedding step (Bobrow et al. 1989). After resin embedding, deposited haptens are then visualised by post-embedding labelling. Free floating vibratome sections were treated according to standard ABC-peroxidase/DAB protocols. After washing in PBS, deposition of TMR was performed in a solution of 10 µM TMRT (2 µM for fluorescence analysis), 10 mM imidazole and 0.0015% H_2O_2 in 50 mM Tris/HCl buffer, pH 7.4. Sections were developed for 15 min. For fluorescence analysis, sections were mounted on gelatine-coated slides and coverslipped. For EM, labelled sections were embedded in araldite.

Post-embedding labelling

Briefly, semithin sections (500 nm) were mounted on aminosilane-coated slides. Etching with sodium methoxide was followed by washing and incubating in 2% H_2O_2. Sections were pre-incubated with 10% NGS, followed by incubation with rabbit-anti-TMR antibody (Molecular Probes). Bound antibodies were visualised according to standard ABC-peroxidase/DAB protocols. Using the same antibody, thin sections (70 nm) were treated as previously described for epoxy-embedded material (Ottersen 1987).

Results

The expression pattern of Agm-like immunoreactivity in the rat brain not only demonstrates a widespread distribution of the enzyme, but also exhibits a broad spectrum in labelling intensity ranging from very intense labelling in several interneuron populations and moderate labelling in principal neurons to relatively weak immunoreactivity in most neuropil areas. Within the cerebral cortex, the whole range in labelling strength is clearly evident even in light micrographs taken from epoxy-embedded 50-µm vibratome sections used for DAB-based electron microscopy (Fig. 1a). While interneurons occurring in different cortical layers were robustly labelled, principal neurons, like layer V pyramidal cells, displayed intermediate labelling intensity. In this preparation as well as in 20-µm cryostat sections (Fig. 1b, c), the neuropil appears less intensely and diffusely labelled, but also shows gradual differences in labelling intensity on comparing layers I/II with layers III–VI. At higher resolution using 1-µm confocal sections obtained from TMR-CARD labelled vibratome preparations, in the neuropil scattered immunoreactive punctate profiles were observed (Fig. 1d). With VirP on 0.5-µm semithin resin sections (Fig. 1e), the neurochemical resolution of neuropil structures was further increased, revealing numerous punctate profiles, either scattered in the neuropil or associated with cell somata and dendrites, the latter putatively representing synaptic terminals. To identify the nature of labelled punctate profiles, we then performed standard peroxidase/DAB-based labelling for electron microscopy. Here, though intensely labelled cell bodies were clearly identified, the labelling in the neuropil was rather subtle and often did not allow to unequivocally identify immunopositive profiles. However, very rarely postsynaptic labelling at dendritic spines was

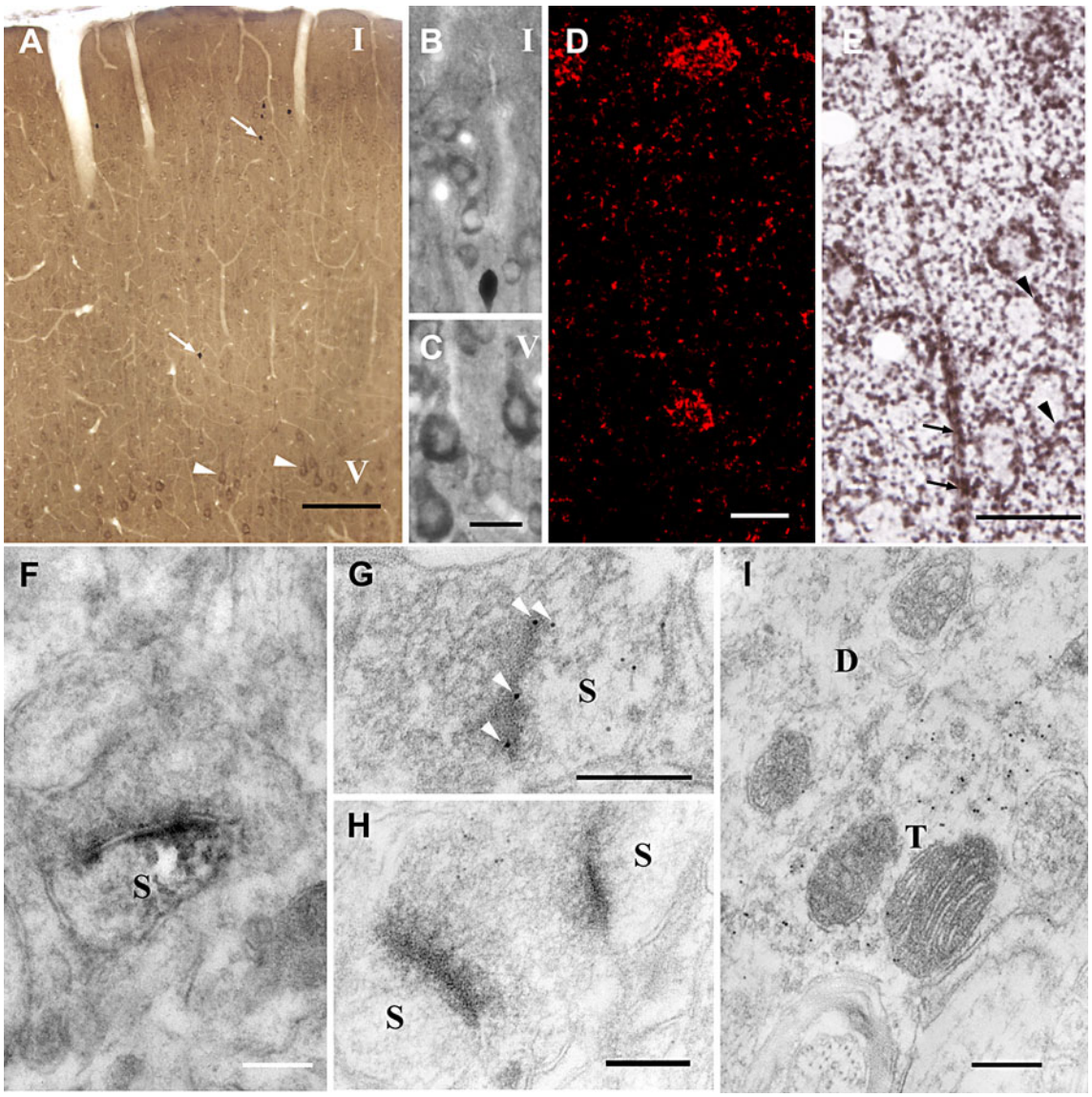

Fig. 1 Agmatinase immunoreactivity in rat parietal cortex. **a** In an osmicated, epoxy-embedded vibratome section (50 μm), cell bodies of heavily DAB-labelled putative interneurons (*arrows*) and moderately labelled principal neurons (*arrowheads* in layer V) are clearly visualised. Neuropil labelling is most pronounced in layers I/II. **b, c** In the neuropil, at high magnification the labelling appears largely diffuse throughout the cortical layers. **d** When increasing the sensitivity using a TMR-based CARD signal amplification, confocal optical sections (1 μm) revealed scattered immunoreactive punctate profiles. **e** With DAB-VirP on semithin sections, the number of immunoreactive punctate profiles in the neuropil was markedly increased when compared to **d**. Aside from scattered profiles, an association with dendrites (*arrows*) and cell bodies (*arrowheads*) was also observed. **f** Electron microscopy of standard DAB-labelled sections only rarely revealed clearly detectable labelling associated with structures likely to correspond to the observed punctate profiles, like dendritic spines (**s**). **g, h, i** With VirP, however, postsynaptically labelled spines (**g**) were frequently observed. **h** By contrast, some immunonegative spines were associated with Agm-containing terminals (**t**). **i** Synaptic vesicle-containing profiles not associated with dendritic spines were also detected. *Scale bars* = 200 μm in **a**, 20 μm in **b, c** and **e**, 10 μm in **d**, and 200 nm in **f–i**

unambiguously displayed (Fig. 1f). To further increase sensitivity and make use of the advantages of particulate marker systems, we performed VirP with rabbit-anti-TMR and 10-nm colloidal gold anti-rabbit antibodies applied in the final post-embedding step (Fig. 1g, h, i). Here, we frequently observed clearly labelled dendritic spines (Fig. 1g). However, labelling was detected only in a minority of asymmetrical spine synapses. Among labelled spines, there was an apparent tendency of gold particles being localised towards the postsynaptic density (arrowheads in g). Whenever postsynaptic labelling in dendritic spines was observed, the respective presynaptic terminals were either devoid of any Agm-like immunoreactivity or, occasionally, showed a comparatively weak immunosignal

when located at sites near the surface of vibratome sections, and thus in regions generally offering optimal labelling sensitivity. Vice versa, some terminals associated with dendritic spines were significantly labelled (Fig. 1h), while the respective postsynaptic compartment did not display significant immunosignal. A presynaptic labelling was also observed in terminals not associated with dendritic spines (Fig. 1i). A cytosolic labelling was frequently observed in dendritic profiles (not shown).

Discussion

Agmatine was proposed to act as a neurotransmitter in the mammalian brain (Li et al. 1994). Morphological (Gorbatyuk et al. 2001) as well as physiological (Goracke-Postle et al. 2007) evidence support this initial presumption. As agmatine persists in the brain with a half-life of approximately 12 h (Roberts et al. 2005), neuromodulatory effects could be effectively terminated by the action of Agm. We therefore postulated that the fine structural localisation of the enzyme may not only further support a synaptic role for agmatine (Bernstein et al. 2011), but also may help to identify brain circuits involved with agmatine neurotransmitter/neuromodulatory action. The data presented here demonstrating the expression of Agm in pre- and postsynaptic compartments of the rat cerebral cortex indeed support the proposed role for Agm as a local inactivator of the neurotransmitter/neuromodulator agmatine. A local inactivation of agmatine was also suggested in the context of mood disorders (Bernstein et al. 2012). Interestingly, Agm immunoreactivity was observed in putative synaptic terminals in the human hippocampus (Bernstein et al. 2012). We now unequivocally demonstrate that Agm is expressed in rat cerebral cortical terminals, thus supporting a presynaptic role for Agm. In spine synapses, however, Agm expression was predominantly localised to postsynaptic compartments, though also detected in some terminals synapsing on dendritic spines. This largely mutually exclusive localisation not only argues in favour of the specificity of the observed immunolabelling, but also strongly supports a modulatory role for an agmatinergic neurotransmission in the cerebral cortex. Furthermore, our data suggest an intracellular enrichment of Agm close to the postsynaptic density. Although a molecular interaction of Agm with PSD scaffolding proteins has so far not been reported, it was shown that in rat hippocampal neurons, agmatine selectively modulates the NMDA subclass of glutamate receptor channels by interacting with the channel pore (Yang and Reis 1999).

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Conflict of interest The authors declare no conflict of interests.

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