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COMPLEMENTARY SYNAPTIC DISTRIBUTION OF ENZYMES RESPONSIBLE FOR SYNTHESIS AND INACTIVATION OF THE ENDOCANNABINOID 2-ARACHIDONOYLGLYCEROL IN THE HUMAN HIPPOCAMPUS

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Abstract—Clinical and experimental evidence demonstrates that endocannabinoids play either beneficial or adverse roles in many neurological and psychiatric disorders. Their medical significance may be best explained by the emerging concept that endocannabinoids are essential modulators of synaptic transmission throughout the central nervous system. However, the precise molecular architecture of the endocannabinoid signaling machinery in the human brain remains elusive. To address this issue, we investigated the synaptic distribution of metabolic enzymes for the most abundant endocannabinoid molecule, 2-arachidonoylglycerol (2-AG), in the postmortem human hippocampus. Immunostaining for diacylglycerol lipase-α (DGL-α), the main synthesizing enzyme of 2-AG, resulted in a laminar pattern corresponding to the termination zones of glutamatergic pathways. The highest density of DGL-α-immunostaining was observed in strata radiatum and oriens of the cornu ammonis and in the inner third of stratum moleculare of the dentate gyrus. At higher magnification, DGL-α-immunopositive puncta were distributed throughout the neuropil outlining the immunonegative main dendrites of pyramidal and granule cells. Electron microscopic analysis revealed that this pattern was due to the accumulation of DGL-α in dendritic spine heads. Similar DGL-α-immunostaining pattern was also found in hippocampi of wild-type, but not of DGL-α knockout mice. Using two independent antibodies developed against monoacylglycerol lipase (MGL), the predominant enzyme inactivating 2-AG, immunostaining also revealed a laminar and punctate staining pattern. However, as observed previously in rodent hippocampus, MGL was enriched in axon terminals instead of postsynaptic structures at the ultrastructural level. Taken together, these findings demonstrate the post- and presynaptic segregation of primary enzymes responsible for synthesis and elimination of 2-AG, respectively, in the human hippocampus. Thus, molecular architecture of the endocannabinoid signaling machinery supports retrograde regulation of synaptic activity, and its similar blueprint in rodents and humans further indicates that 2-AG's physiological role as a negative feed-back signal is an evolutionarily conserved feature of excitatory synapses.

Key words: 2-arachidonoylglycerol, diacylglycerol lipase, monoacylglycerol lipase, CB_1 cannabinoid receptor, glutamatergic synapse, hippocampus.

Various preparations of the leaves, flowers and resinous extracts of the Cannabis plant have been consumed for both medical and recreational purposes since antiquity. Extensive research efforts aiming to explain the widespread therapeutic and behavioral effects evoked by Cannabis consumption finally led to the discovery of a new messenger system called the endocannabinoid system. It is composed of the CB_1 and CB_2 cannabinoid receptors (Devane et al., 1988; Matsuda et al., 1990; Munro et al., 1993), which are not only targets of the psychoactive compounds of the Cannabis plant, but more importantly, they are also activated by two endogenous lipid molecules produced by most cell types in the body (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). The mobilization and elimination of these two endocannabinoid molecules called *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) are tightly regulated by surprisingly complex networks of metabolic enzymes and pathways in different cell types and tissues (Piomelli, 2003; Alexander and Kendall, 2007; Ahn et al., 2008). Following the clinical failure of brain-penetrating CB_1 receptor antagonists as therapeutics due to adverse psychiatric effects, the identification of novel molecular players regulating endocannabinoid levels has opened new possibilities, because drugs targeting these enzymes may have more selective actions (Ahn et al., 2009; Bisogno et al., 2009; Long et al., 2009; Solorzano et al., 2009). The wide spectrum of human neurological and psychiatric diseases in which the endocannabinoid system is implicated suggests a vast therapeutic potential (Mackie,

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Abbreviations: DAB, 3,3-diaminobenzidine; DGL-α, diacylglycerol lipase-α; MGL, monoacylglycerol lipase; $mGluR_5$, metabotropic glutamate receptor type 5; PB, phosphate buffer; PLC-β, phospholipase C-β; TBS, tris-buffered saline; 2-AG, 2-arachidonoylglycerol; Δ^9-THC, Δ^9-tetrahydrocannabinol.

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2006; Pacher et al., 2006; Katona and Freund, 2008). However, to take advantage of this potential requires full characterization of the enzymes regulating endocannabinoid signaling in the human brain.

A conceptual framework describing the major aspects of neuronal endocannabinoid signaling has emerged from the results of numerous animal studies in the last decade. In contrast to conventional neurotransmitters, endocannabinoids are primarily synthesized and released by postsynaptic neurons in an on-demand manner (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001), and subsequently activate presynaptically located CB_1 cannabinoid receptors, thereby regulating neurotransmitter release from several types of axon terminals (Freund et al., 2003; Kano et al., 2009). This retrograde manner of synaptic endocannabinoid signaling is indispensable for various forms of homo- and heterosynaptic plasticity throughout the central nervous system (Chevaleyre et al., 2006; Kano et al., 2009), and probably accounts for the extensive involvement of the endocannabinoid system in brain disorders (Katona and Freund, 2008). Thus, molecular mechanisms regulating synaptic endocannabinoid signaling may be of pivotal importance in the therapeutic exploitation of the endocannabinoid system.

Although anandamide is the archetypical endocannabinoid molecule (Devane et al., 1992), and may tonically control presynaptic CB_1 receptors (Kim and Alger, 2010), most experimental evidence converge on the notion that 2-AG is the crucial retrograde messenger mediating on-demand forms of short- and long-term synaptic depression through CB_1 activation. Pharmacological inhibition of diacylglycerol lipase, including its alpha isoform (DGL-α), the enzyme primarily responsible for 2-AG biosynthesis in adult brain (Bisogno et al., 2003), prevents retrograde endocannabinoid signaling in various experimental paradigms throughout the cerebral cortex (Chevaleyre and Castillo, 2003; Straiker and Mackie, 2005; Edwards et al., 2006, 2008; Lafourcade et al., 2007; Hashimotodani et al., 2008; Kellogg et al., 2009; Zhang et al., 2009, but see Min et al., 2010). Particularly compelling support for this concept also derives from the genetic inactivation of DGL-α, which completely abolishes endocannabinoid-mediated synaptic plasticity, for example in the hippocampus (Gao et al., 2010; Tanimura et al., 2010). Conversely, pharmacological blockade of monoacylglycerol lipase (MGL), the enzyme responsible for inactivation of the major fraction ($\sim$85%) of 2-AG in the brain (Dinh et al., 2002; Blankman et al., 2007), prolongs retrograde endocannabinoid signaling in distinct types of synapses (Makara et al., 2005; Hashimotodani et al., 2007; Lafourcade et al., 2007; Pan et al., 2009; Straiker et al., 2009; Straiker and Mackie, 2009; Zhang et al., 2009).

Widespread distribution of 2-AG in the human brain has recently been revealed (Palkovits et al., 2008) with a largely overlapping regional pattern to CB_1 receptors based on radioligand binding and *in situ* hybridization experiments (Herkenham et al., 1990; Westlake et al., 1994; Glass et al., 1997). Further high-resolution immunostaining and electron microscopic analysis in the human hippocampal formation and neocortex have narrowed down the presence of CB_1 receptors to GABAergic boutons (Katona et al., 2000; Eggan and Lewis, 2007; Ludanyi et al., 2008; Eggan et al., 2010; Magloczky et al., 2010) and also to glutamatergic axon terminals (Ludanyi et al., 2008). Together, these findings contribute to the hypothesis that 2-AG may be a synaptic messenger in the human nervous system. However, despite their potential therapeutic significance and their prominent mRNA expression levels in the human hippocampus (Ludanyi et al., 2008), the precise location of two key enzymes, DGL-α and MGL, known to regulate 2-AG signaling at chemical synapses in rodents have not yet been investigated in detail in the human brain. The aim of our study was therefore to uncover the precise molecular organization of the 2-AG signaling pathway at excitatory synapses in the human hippocampus by using novel antibodies with confirmed target specificity for DGL-α and MGL, as well as light and high-resolution electron microscopy.

EXPERIMENTAL PROCEDURES

Human tissue samples

Control hippocampi (n=5) were kindly provided by the Lenhossék Human Brain Program, Semmelweis University, Budapest. Control subjects (57$\pm$3 years) died suddenly from causes not directly involving any brain disease, and none of them had a history of any neurological disorders. The control subjects were processed for autopsy in the Department of Forensic Medicine of the Semmelweis University Medical School, Budapest, and the brains were removed 2–5 h after death. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. All procedures were approved by the Regional and Institutional Committee of Science and Research Ethics of Scientific Council of Health (TUKEB 5-1/1996).

After postmortem removal, the hippocampal tissue was immediately dissected into 3- to 4-mm-thick blocks, and immersed in a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.2% picric acid in phosphate buffer (PB; pH 7.4; 0.1 M). The blocks were first rinsed for 6 h at room temperature in the fixative, which was replaced every hour with a fresh solution. The blocks were then postfixed overnight in the same fixative solution, but without glutaraldehyde. In the case of one control brain, both the internal carotid and vertebral arteries were cannulated 4 h after death, and the brain was perfused with physiological saline (2 L in 30 min) followed by a fixative solution containing 4% paraformaldehyde and 0.2% picric acid in PB (5 L in 3.5 h). The hippocampus was removed after perfusion, and was cut into 3- to 4-mm-thick blocks, and was postfixed in the same fixative solution overnight. Subsequently, 60-μm-thick coronal sections were prepared from the blocks with a Leica VTS-1000 Vibratome (Leica Microsystems, Wetzlar, Germany) for immunohistochemistry.

Mouse tissue samples

Adult male DGL-α knockout mice and wild-type littermates on C57BL/6 background (Tanimura et al., 2010) for the immunoperoxidase experiment as well as male wild-type C57BL/6 mice for the immunofluorescence experiment were perfused transcardially under deep pentobarbital (Phylaxia-Sanofi, Budapest, Hungary) anesthesia first with 0.9% saline and then with 4% paraformaldehyde dissolved in PB. After perfusion, the brain was removed from the skull, cut into 3- to 4-mm-thick blocks, and then 50-μm-thick