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Invited review

More surprises lying ahead. The endocannabinoids keep us guessing

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ABSTRACT

The objective of this review is to point out some important facts that *we don't know* about endogenous cannabinoids — lipid-derived signaling molecules that activate CB_1 cannabinoid receptors and play key roles in motivation, emotion and energy balance. The first endocannabinoid substance to be discovered, anandamide, was isolated from brain tissue in 1992. Research has shown that this molecule is a *bona fide* brain neurotransmitter involved in the regulation of stress responses and pain, but the molecular mechanisms that govern its formation and the neural pathways in which it is employed are still unknown. There is a general consensus that enzyme-mediated cleavage, catalyzed by fatty acid amide hydrolase (FAAH), terminates the biological actions of anandamide, but there are many reasons to believe that other as-yet-unidentified proteins are also involved in this process. We have made significant headway in understanding the second arrived in the endocannabinoid family, 2-arachidonoyl-*sn*-glycerol (2-AG), which was discovered three years after anandamide. Researchers have established some of the key molecular players involved in 2-AG formation and deactivation, localized them to specific synaptic components, and showed that their assembly into a multi-molecular protein complex (termed the '2-AG signalosome') allows 2-AG to act as a retrograde messenger at excitatory synapses of the brain. Basic questions that remain to be answered pertain to the exact molecular composition of the 2-AG signalosome, its regulation by neural activity and its potential role in the actions of drugs of abuse such as Δ^9-THC and cocaine.

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When anandamide was isolated from pig brain in 1992 and identified as the first endogenous marijuana-like substance (Devane et al., 1992), the neuroscience community was quick to realize the significance of this finding (Barinaga, 1992). Only two years earlier, the molecular characterization of a cell-surface receptor that recognizes marijuana's main psychoactive constituent, Δ^9-tetrahydrocannabinol (Δ^9-THC)(Devane et al., 1988; Matsuda et al., 1990), had finally answered the longstanding question of how this compound exerts its unique brand of pharmacological effects — a combination of euphoria, calmness, appetite stimulation, sensory alterations and analgesia (Iversen, 2000). The isolation of anandamide promised to unlock the door to the brain neurotransmitter system hijacked by Δ^9-THC. Past experience with peptide transmitters suggested that this expectation was not unrealistic: for example, the identification of the enkephalins (Hugues et al., 1975) and their receptors (Pert and Snyder, 1973; Terenius, 1973) had quickly led to the anatomical mapping of opioidergic pathways in the central nervous system (CNS) (Pert et al., 1976), a key step toward uncovering the roles of opioid peptides in the regulation of brain function and behavior. Therefore, optimism that similar advances would ensue from the discovery of anandamide was reasonably justified.

Indeed, a great deal has been learned in the years following that discovery: there is now convincing evidence that anandamide is a *bona fide* neurotransmitter (Di Marzo et al., 1994; Giuffrida et al., 1999) involved in the control of synaptic plasticity in the amygdala (Puente et al., 2011), the modulation of stress responses (Bortolato et al., 2007; Dlugos et al., 2012; Gobbi et al., 2005; Gunduz-Cinar et al., 2012; Hill et al., 2010; Kathuria et al., 2003), and the processing of central and peripheral pain signals (Hohmann et al., 2005; Clapper et al., 2010). Despite this progress,

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however, we still don't fully understand how anandamide is produced in neurons, in which neural pathways it acts as a neurotransmitter, and what physiological stimuli trigger its release.

Why has anandamide proved to be such a hard nut to crack? How does this lipid-derived molecule differ from 2-arachidonoyl-*sn*-glycerol (2-AG), a more recent addition to the endocannabinoid lipid family (Mechoulam et al., 1995; Sugiura et al., 1995) for which we have obtained, within a few years of its discovery, a reasonably detailed knowledge of metabolism (Bisogno et al., 2003;; Stella et al., 1997) and synaptic localization (Katona et al., 2006; Nyilas et al., 2009)? Answering this question may provide important insights, not only on the biochemical properties and signaling functions of anandamide, but also on endocannabinoid transmission at large.

1. 2-AG formation – a new trick for an old dog

Neurons produce 2-AG through a multifunctional lipid pathway that starts with the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) and ends (at least temporarily) with the transient accumulation of non-esterified arachidonic acid in cell membranes (Fig. 1). Individual checkpoints along this route can each generate separate sets of signaling molecules (for review, see Piomelli et al., 2007).

The first checkpoint is represented by the β–isoform of phospholipase C (PLC-β), which can be activated by various G protein-coupled receptors (e.g. the type-1 metabotropic glutamate receptor, mGluR5) and converts PIP_2 into the second messenger 1,2-diacylglycerol (DAG)(Bennet et al., 1988). DAG regulates the activity of protein kinase C and other cellular effectors, but also serves as substrate for two functionally distinct enzymes: diacylglycerol kinase, which generates the intracellular signaling lipid (and metabolic intermediate), phosphatidic acid; and diacylglycerol lipase-α (DGL-α), which hydrolyses DAG forming 2-AG (Bisogno et al., 2003; Stella et al., 1997). In addition to serving as an endocannabinoid agonist, 2-AG can be also oxygenated by cyclooxygenase-2 (Cox-2) to yield a family of prostaglandin (PG) glyceryl esters, which do not engage cannabinoid receptors yet display significant biological activities (Kozak et al., 2000). Moreover, 2-AG can be cleaved by hydrolases such as monoacylglycerol lipase (MGL) (Dinh et al., 2002) to produce non-esterified arachidonic acid (Fig. 1). Like other polyunsaturated fatty acids, free arachidonate is either immediately reinserted into membrane phospholipids (part of a process known as 'phospholipid remodeling') or utilized for the production of eicosanoids (e.g., Cox-2-derived prostaglandins) (Piomelli et al., 2007).

The key reactions in the multifunctional pathway outlined above – and particularly the sequential contributions of PLC, DGL and MGL activities to the release of arachidonic acid from membrane phospholipids – have been known for decades (Allen et al., 1992; Bell et al., 1979). This prior knowledge greatly facilitated the molecular cloning of the main enzymes involved in 2-AG production (Bennet et al., 1988; Bisogno et al., 2003; Dinh et al., 2002), which in turn made possible the identification of the supramolecular protein complex ('signalosome') that enables 2-AG signaling at excitatory synapses of the brain (Jung et al., 2012a). We will come back to the possible functions of the 2-AG signalosome later on in this article.

2. The trouble with anandamide

In contrast with 2-AG, the reactions leading to the production of anandamide are relatively unprecedented in lipid biochemistry. This state of affairs is not surprising, if we consider that anandamide and other members of its chemical family – the amides of ethanolamine with long-chain fatty acids (known as *N*-acylethanolamines or fatty acid ethanolamides [FAEs]) – were initially dismissed as being terminal products of *post mortem* tissue degradation rather than physiologically meaningful signaling molecules (Schmid et al., 1995; Kempe et al., 1996). Indeed, the functional significance of anandamide remained controversial until the mechanisms underlying the production and deactivation of this compound were outlined using primary cultures of rat brain neurons (Cadas et al., 1996, 1997; Di Marzo et al., 1994) and its activity-dependent release in the CNS was demonstrated by microdialysis in freely moving rats (Giuffrida et al., 1999).

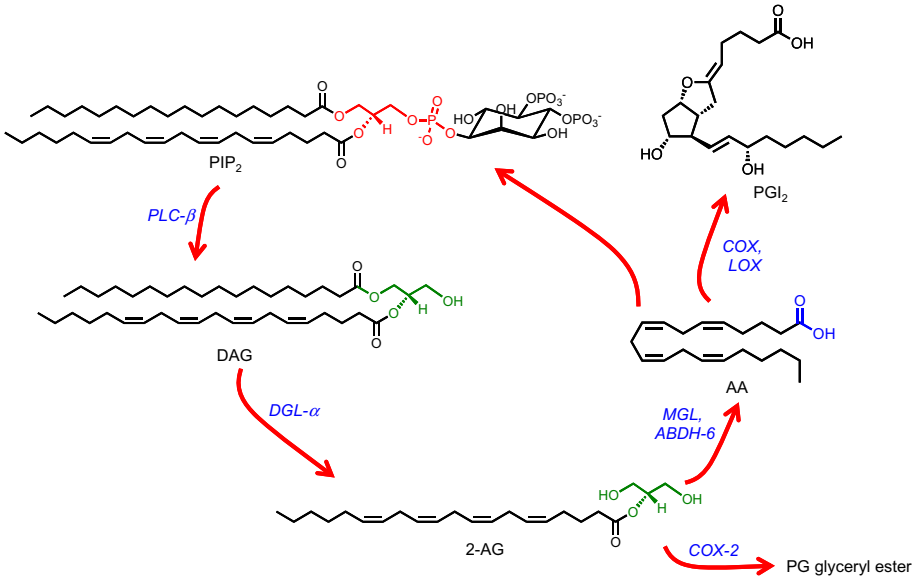

Fig. 1. Formation and deactivation of 2-AG in brain neurons. Receptor-operated phospholipase C-β (PLC-β) converts phosphatidylinositol-4,5-bisphosphate (PIP2) into 1,2-diacylglycerol (DAG). DAG is hydrolyzed by diacylglycerol lipase-α (DGL-α) forming 2-AG. 2-AG is subjected to hydrolytic cleavage catalyzed by either monoacylglycerol lipase (MGL) or α/β hydrolase domain-containing protein 6 (ABHD-6). Additionally, 2-AG can be oxygenated by cyclooxygenase-2 (Co_x-2) to yield a family of non-endocannabinoid prostaglandin (PG) glyceryl esters. Free arachidonic acid (AA) is converted into the eicosanoid family of compounds, for example prostacyclin (PGI2), by cyclooxygenase or lipoxygenase enzymes.